Regulation of nitric oxide and chemokine production in human colonic epithelial cells: implication in intestinal inflammation

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REGULATION OF NITRIC OXIDE AND CHEMOKINE PRODUCTION IN HUMAN COLONIC EPITHELIAL CELLS: IMPLICATION IN INTESTINAL INFLAMMATION

submitted by George C. Kolios

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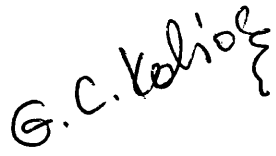

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ABSTRACT

Colonic epithelium represents an important interface between the host and external environment serving both as a surface for absorption and a defence against ingested pathogens. Colonic epithelial cells may play an important role in inflammatory and immune reactions and participate in the communication between inflammatory and immune cells, probably via the generation of inflammatory mediators. The potential of human colonic epithelial cells to produce inflammatory mediators such as chemokines and nitric oxide and the modulation of this production were examined.

Colonic epithelial cells HT-29 were found to produce nitric oxide (NO) after stimulation with specific combinations of the pro-inflammatory cytokines IL-1α, TNF-α and IFN-γ. This NO generation was via the induction of the inducible nitric oxide synthase (iNOS), since iNOS mRNA and protein expression were observed after stimulation with cytokines. The T cell derived anti-inflammatory cytokines, IL-4 and IL-13 reduced significantly this NO generation and iNOS expression, while the other T cell derived cytokine, IL-10, had not a similar effect. Immunohistochemical study of human colonic biopsies revealed iNOS expression in colonic epithelial cells from patients with ulcerative colitis (UC) and infectious colitis, while the iNOS expression was absent in normal colonic mucosa or in colonic mucosa from infectious colitis patients in total remission.

Pro-inflammatory cytokines were found to induce chemokine expression in HT-29 cells. Stimulated cultures of HT-29 cells produced the CXC chemokine, IL-8, and the CC chemokines MCP-1 and RANTES and expressed IL-8, MCP-1, and RANTES mRNA. IL-13 and IL-4 were found to have a differential effect on CXC and CC chemokine generation by HT-29 cells, reducing significantly the MCP-1 and RANTES, but not the IL-8 expression and production. In addition, in immunohistochemical studies the neutrophil chemoattractant IL-8 was found expressed in colonic epithelial cells and lamina propria cells in inflamed mucosa from UC and infectious colitis patients, while IL-8 expression was not found in normal colonic mucosa.

LIST OF FIGURES AND TABLES

ABBREVIATIONS

ABC	Avidin-Biotin complex
AcD	Actinomycin D
ADP	Adenosine diphosphate
ANOVA	Analysis of variance
AP-1	Activator protein-1
APES	3-Aminopropyltriethoxysilane
APS	Ammonium persulphate solution
5-ASA	5-Aminosalysilate
ATP	Adenosine triphosphate
bFGF	Basic fibroblast growth factor
BH_4	Tetrahydropterin
BSA	Bovine serum albumin
cAMP	Cyclic adenosine-5'-monophosphate
CCR	CC chemokine receptor
cGMP	Cyclic guanisine-5'-monophosphate
CD	Crohn's disease
CHO	Chinese hamster ovary cells
CHX	Cycloheximide
CINC	Cytokine-induced neutrophil chemoattractant (rat homologue of IL-8)
cNOS	Constitutive nitric oxide synthase
Con-A	Conconavalin
CSF-1	Colony-stimulating factor-1
CSPD	Disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2'-(5-chloro) tricyclo [3.3.1.^{3,7}] decan}-4-yl) phenyl phosphate
CT	Cholera toxin
CT-B	Cholera toxin B oligomer
CTAP-III	Connective tissue activating protein III
CTP	Cytosine triphosphate
CXCR	CXC chemokine receptor

DAB	3,3' Diaminobenzidine
DAG	Diacyglycerol
DAN	2,3-Diaminonaphthalene
DARC	Duffy antigen receptor for chemokines
Db-cAMP	Dibutyril cyclic adenosine monophosphate
DEPC	Diethyl pyrocarbonate
DIG	Digoxigenin
DMEM	Dulbecco's modified Eagles medium
DMF	Dimethylformamide
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGF	Epithermal growth factor
ELISA	Enzyme-linked immunosorbant assay
ENA-78	Epithelia-derived neutrophil attractant-78
FAD	Flavin dinucleotide
FCS	Foetal calf serum
FITC	Fluorescein isothiocynate
FMLP	N-formyl-methionyl-leucyl-phenylalanine
FMN	Flavin mononucleotide
GBM	Glomural basement membrane
GCP-2	Granulocyte chemotactic protein-2
GDP	Guanosine diphosphate
GM-CSF	Granulocyte/macrophage colony-stimulating factor
GN	Glomerulonephritis
GTP	Guanosine triphosphate
HBSS	Hanks balanced salt solution
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethane sulphonic acid
HLA	Human leukocyte antigen
HLA-DR	D-related HLA

HMAP	4-hydroxy-3-methoxyaceto-phenone
HX	Haematoxyline
IBD	Inflammatory bowel disease
IBMX	3-isobutyl-l-methyl-xanthine
IEL	Intraepithelial lymphocytes
IFN-γ	Interferon-γ
IGF-1	Insulin-like growth factor-1
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
iNOS	Inducible nitric oxide synthase
IP-10	IFN-γ-inducible 10
K-K	Kallikrein-kinin
LFA-1	Leukocyte function antigen-1
L-NAA	N^{ω}-amino-L-arginine
L-NMMA	N^{ω}-monomethyl-L-arginine
L-NNA	N^{ω}-nitro-L-arginine
LPS	Lipopolysaccharide
LT	Leukotriene
MAP kinase	Mitogen-activated protein kinase
MC	Mesangial cell
MCP-1	Monocyte chemotactic protein-1
MGSA/gro	Melanocyte growth stimulatory activity
MHC	Major histocompatibility complex
mig	Monokine induced by IFN-γ
MIP-1α	Macrophage inflammatory protein-1α
MOPS	3-[N-morpholino]propane-sulphonic acid
mRNA	Messenger ribonucleic acid
MUC	Mucin
NAD	Nicotinamide adenine dinocleotide

NADPH	Nicotinamide adenine dinocleotide phosphate
NAP-2	Neutrophil activating peptide-2
NF-κB	Nuclear factor- κB
NO	Nitric oxide
NOS	Nitric oxide synthase(s)
NSAID	Non-steroid anti-inflammatory drug
NTN	Nephrotoxic serum nephritis
OD	Optical density
OPD	O-phenylenediamine dihydrochloride
[^{32}P]	Phosphate radiolabel
PAF	Platelet activating factor
p-ANCA	p-antineutrophil cytoplasmic antibodies
PBP	Platelet basic protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGF	Platelet-derived growth factor
PDTC	Pyrrolidinedithiocarbonate
PF4	Platelet factor 4
PGE_2	Prostaglandin E_2
PGI_2	Prostacyclin
PKA	Protein kinase A
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLC	Phospholipase C
PMFC	Phenylmethylsulphonyl fluoride
PSTI	Pancreatic secretory trypsin inhibitor
PT	Pertussis toxin
PT-B	Pertussis toxin B oligomer
RANTES	Regulated on activation, normal T cell expressed and secreted
RIA	Radioimmunoassay

ROS	Reactive oxygen species
SC	Secretory component
SCFA	Short chain fatty acids
SDF-1α	Stromal cell-derived factors 1α
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SIgA	Secretory IgA
SOD	Superoxide dismutase
SOM	Somatostatin
SP	Substance P
TARC	Thymus and activation related chemokine
TBS	Tris buffered saline
TCR	T cell receptor
TEMED	N, N, N', N'-tetramethylethylene diamine
β-TG	β-thromboglobulin
TGFβ	Transforming growth factor-β
TNF	Tumour necrosis factor
TNF-R1	Tumour necrosis factor receptor 1
TNF-R2	Tumour necrosis factor receptor 2
TRH	Thyrotropin releasing hormone
Tween-20	Polyoxyethylenesorbitan monolaurate
TXA_2	Thromboxane A_2
TXB_2	Thromboxane B_2
UC	Ulcerative colitis
VIP	Vasoactive intestinal polypeptide
VLA_4	Very late antigen-4

1. INTRODUCTION

1. 1 IMMUNOLOGICAL ROLE OF COLONIC EPITHELIUM

Colonic mucosa is the innermost layer of the large bowel wall. It consists of the colonic epithelium, which covers the absorptive surface and the crypts, and of the lamina propria, which is found immediately beneath the epithelium. Colonic epithelium is composed of colonic epithelial cells, that represent the main population, mucus secreting goblet cells and endocrine epithelial cells. Intraepithelial lymphocytes infiltrate throughout the epithelium. The primary function of the intestinal epithelium is the absorption of nutrients, water and electrolytes from the lumen to the body. However, during the last decades it has been realised that the epithelium itself is involved in various immunological and inflammatory processes.

The discovery of the molecular basis of how IgA antibodies function on the surface of epithelial cells demonstrated a new role of the intestinal mucosa. Tomasi in 1970 showed that mucosal IgA was synthesized by plasma cells as a dimer, which combine with secretory component (SC), a glycopeptide product of epithelial cells, to form secretory IgA (SIgA) (Tomasi, 1970). SC is a transmembrane polypeptide, which is preferentially expressed on the basolateral aspects of intestinal epithelial cells. This peptide protects the IgA dimer from luminal proteolysis (Mostov *et al.* 1984). In 1973 Brandtzaeg reported that IgM was also adapted to function at mucosal surfaces by binding SC to form secretory IgM (Brandtzaeg, 1973). The functional importance of SIgA was first shown in children vaccinated by the oral Sabin poliovirus vaccine. The intestinal epithelium represents an important interface between the host and external environment serving both as a surface for absorption and a defence against ingested pathogens (McKay & Perdue, 1993a; McKay & Perdue, 1993b). Both non-immune and immune mechanism protect the privileged environment of the lamina propria from challenge by foreign antigens.

Intestinal motility, commensal microflora and mucous coat or glycocalyx comprise some of the non-specific protective barriers. The immune mechanisms may operate within the lumen of the gut, at the mucosal surface or within the lamina propria (Abreumartin & Targan, 1996). The intestinal epithelial cells constitute a barrier between the environment and the host tissue and they are the first cells to come in contact with many pathogens. From this position intestinal epithelial cells might play a crucial role as an outpost of the immune system located in the underlying intestinal mucosa and soluble mediators produced by these cells might function as an early signal to neighbouring immune cells and are involved in the recruitment of cells during the inflammatory response. Finally, the interplay between the colonic epithelial cells and intraepithelial lymphocytes (IEL) may complete the immunological role of colonic mucosa.

It is thought that IEL may play a central role in local intestinal immunity and are likely to be important in immunity to gastrointestinal neoplasms and rejection responses to gut allografts. However, the biology of IEL and their unusual immunological microenvironments in the gastrointestinal mucosa are little understood (Croitoru & Ernest, 1992; Cerf-Benussan & Gay-Grand, 1991). The IEL comprise two populations. The predominant group is the $CD8^+$ suppressor-cytolytic subset of T lymphocytes (Ts) which display the α/β T cell receptor (TCR) and show evidence of activation. The second group of T cells bears the γ/δ form of TCR. These T cells are mostly $CD4^-$. Most of the lamina propria T lymphocytes belong to the $CD4^+$ helper-inducer subset (Th) and γ/δ cells are rare (Trejdosiewicz, 1992).

A role for colonic epithelial cells in inflammatory and immune reactions is increasingly recognised. These cells present antigens via class II molecule expression and act as antigen presenting cells to T cells (Mayer *et al.* 1991; Lowes *et al.* 1992). Class II MHC determinants are expressed on normal small intestinal epithelial cells but not on colon epithelial cells unless the colon is inflamed (Selby *et al.* 1983). Colonic epithelial cells in

Gαi-deficient mice with colitis were found by immunohistochemistry to express increased levels of both MHC class I and class II antigens (Hornquist *et al.* 1997). Expression of Class II antigens may be modulated by IFN-γ produced by activated $CD8^+$ T cells in the epithelium (Cerf-Bensussan *et al.* 1984). The expression of HLA molecules by colonic epithelial cells was found to activate $CD3^+/CD8^+$ IEL (Hoang *et al.* 1992). Colonic epithelial cells express adhesion proteins such as intercellular adhesion molecule-1 (Kaiserlian *et al.* 1991; Kvale & Brandtzaeg, 1995) and generate soluble inflammatory mediators e.g. arachidonic acid derivatives (Gustafson & Tagesson, 1990; Dias *et al.* 1992), platelet activating factor (Ferraris *et al.* 1993), cytokines (Hedges *et al.* 1992; Eckmann *et al.* 1993; Schuerer-Maly *et al.* 1994; Kolios & Nakos, 1995; Gross *et al.* 1995), and chemokines (Mazzucchelli *et al.* 1994; Jung *et al.* 1995), all of which contribute to the communication between inflammatory cells and cells of the immune system (Sartor, 1994). Colon epithelial cells appear to be programmed to provide a set of signals for the activation of the mucosal inflammatory response in the earliest phases after microbial invasion (Jung *et al.* 1995). The involvement of colonic epithelial cells in immune and inflammatory reactions in inflammatory bowel disease is discussed below in detail.

1. 2 INFLAMMATORY BOWEL DISEASE

The term inflammatory bowel disease (IBD) describes two major pathological conditions involving the gastrointestinal tract, ulcerative colitis (UC) and Crohn's disease (CD). In UC the inflammatory disorder is superficial in the mucosa and submucosa and limited to the large bowel, affecting the rectum and a variable extend of the colon. Histologically, the disease is characterised by an infiltration of both acute and chronic inflammatory cells in the lamina propria and the crypts forming microabscesses. The deeper layers of the intestinal wall are characteristically not involved. CD is a transmural inflammation that may occur anywhere throughout the alimentary tract from the mouth to the anus. The disease can affect the deeper layers of the bowel wall producing superficial and deep

ulceration and also leading to collagen deposition and fibrosis. The cellular infiltration consists of lymphocytes and macrophages and the presence of granulomas is a predominant feature of the disease. Both of these diseases are characterised by chronic relapses and associated with many extraintestinal manifestations.

Despite extensive investigation over the last decades the aetiology and pathogenesis of both major forms of IBD remains unknown. Increasing evidence suggests that these two disorders are partly and possibly wholly distinct in their initial aetiological event, but they share important common pathogenetic mechanisms. It is likely that the aetiology of IBD is not a single cause and effect relationship and probably represents an interaction between various agents. A number genetic predisposing factors, exogenous and endogenous triggers and modifying factors is involved and their interaction is an inflammatory process, in which tissue injury seem to be mediated by the immune system (Shanahan, 1993). The prognosis of ulcerative colitis and Crohn's disease is much improved over the years, but an aetiologic cure has not yet been found, re-emphasising the need for further investigation of these challenging diseases. Until the cause of these diseases has been clearly identified therapeutic strategies for these conditions must be based on interrupting the immunopathogenetic mechanisms involved and inhibiting the gut inflammation; hence the widespread use of immunosuppressives in their treatment (MacDonald & Murch, 1994). In the following section, I review the recent clues in aetiology and pathogenesis of IBD, concentrating mostly on immunological mechanisms, since the local immune response probably causes the tissue damage in these diseases.

1. 2. 1 Aetiology of IBD

The cause of IBD is likely to be multifactorial. Although the aetiology of IBD remains obscure, a number of genetic, environmental, microbial, and immunologic factors responsible for the cause of this disorder have been suggested and multiple aetiological theories have been proposed.

Genetics. Genetic influence in the susceptibility to IBD is suggested by racial, ethnic, and familial aggregation of disease (Pena *et al.* 1993). Both CD and UC are familial. Between 10% and 20% of close relatives may also be affected. Patients with CD have a stronger family history than patients with UC. Further support for a stronger genetic influence in Crohn's disease has come from studies of twins (Ellis, 1995). Increased concordance for IBD in monozygotic compared with dizygotic twins suggests that genetic rather than environmental factors are primarily responsible for the familial aggregation. The concordance rate of monozygotic twins with CD is higher than of twins with UC (Tysk *et al.* 1988). Although these observations suggest the existence of genetic factors in the aetiology of IBD they do not support a simple mendelian pattern of inheritance. The search for a specific genetic marker has been disappointing. At present, HLA class II antigens are most in focus, but also genetic markers such as the antineutrophil-cytoplasmic-autoantibodies (p-ANCA) are strongly associated with UC and may represent an indicator of genetic susceptibility to UC (Jarnerot, 1996). The well recognised association of HLA antigens with diseases, as for example the ankylosing spondylitis and sclerosing cholangitis, that occur in association or as complications with IBD is indirect evidence for a genetic relation of these antigens with IBD. Many studies have reported an association of IBD with HLA-DR (Asakura *et al.* 1982; Kobayashi *et al.* 1990; Toyoda *et al.* 1993) and one of them found an even stronger association with DWQ1 (Kobayashi *et al.* 1990). Genes of the major histocompatibility complex are implicated as important inherited determinants of susceptibility to UC and may also influence the pattern of disease. In CD, important susceptibility genes are likely to exist outside the HLA region (Satsangi *et al.* 1996). Recently, it has been have shown that the TNF-α 2b1c2d4e1 allelic combination is the strongest genetic risk factor described in CD and, with HLA class II alleles, defines a group of markers on chromosome 6 that extends from HLA class II to upstream of the TNF-beta gene (Plevy *et al.* 1996). The IL-1ra gene is a marker for genetic susceptibility and severity in UC and contributes to the definition of the

immunogenetic heterogeneity of the disease (Bioque *et al.* 1995; Bioque *et al.* 1996). Future studies using molecular biologic technology with highly informative polymorphic genetic markers should permit successful identification of possible IBD-susceptibility genes.

Diet. It is reasonable to propose that dietary factors, for example acting as antigens, play a role in the initiation of intestinal inflammation, but there is less evidence to support this hypothesis in UC than in CD. In UC, a minority of patients improve on avoidance of cow's milk, but intravenous feeding, which would be expected to prove beneficial, was not found effective, on the other hand, in patients with CD, elemental diets appear to have a therapeutic effect, although the mechanism by which this occurs is uncertain (O'Morain *et al.* 1984; Mishkin, 1997). The role of dietary antigens in the aetiology of CD is controversial. Controlled studies have suggested that elemental diets may be as effective as corticosteroids in inducing a remission in patients with acute CD (Okeefe, 1996), however, corticosteroids are more effective than enteral nutrition in the treatment of active CD (King, 1995). Favourable reports of the value of exclusion diets (Riordan *et al.* 1993), or specific diets (Campbell *et al.* 1997) have not been confirmed by other workers. Patients with elevated yeast antibodies tended to develop a higher CD activity. These results suggest that dietary yeast may affect the activity of CD, but does not necessarily implicate them as aetiological factors (Barclay *et al.* 1992). A population-based case-control study of IBD and dietary habits has shown that the relative risk of CD was increased for subjects who had a high intake of sucrose and was decreased for subjects who had a high intake of fibre (Persson *et al.* 1992). UC patients also consumed more total protein than controls (Tragnone *et al.* 1995). Recently, an enteric-coated preparation of fish oil has been shown to be effective in patients with CD, and it seem prudent to promote a diet rich in fish oil in patients with CD (Kim, 1996).

Infection. A wide variety of infective agents has been suspected to initiate, and/or trigger relapse of IBD. Relapse of UC has been documented in association with proven infection with shigella (Felsen, 1936), salmonella (Lindeman *et al.* 1967), campylobacter jejuni (Newman & Lambert, 1990), clostridium difficile (Bolton *et al.* 1980), aeromonas (Willoughby *et al.* 1989) entamoeba histolytica (Rampton *et al.* 1983) and cytomegalovirus (Berk *et al.* 1985). Pathological resemblances between CD , tuberculosis and John's disease in cattle, which is caused by Mycobacterium paratuberculosis, have evoked intense study of the possible aetiological role of the latter organism (Ciclitira, 1993; Mishina *et al.* 1996), but data from other studies do not support a role for M. paratuberculosis in Crohn's disease (Dumonceau *et al.* 1996; Suenaga *et al.* 1995; Rowbotham *et al.* 1995). Recently a paramixovirus, possibly measles, have been identified in the vascular endothelium of CD intestine (Ciclitira, 1993; Wakefield *et al.* 1993). Recent epidemiological data support the idea that early exposure to measles virus in childhood is a risk factor for the later development of CD (Wakefield, 1995; Thompson *et al.* 1995b; Ekbom *et al.* 1994), but also of UC (Thompson *et al.* 1995a). Furthermore data indicate that exposure of mothers to measles virus in utero is a risk factor for CD in their children (Ekbom *et al.* 1996). These data are consistent with the possibility that CD may be a chronic granulomatous vasculitis in reaction to a persistent infection with measles virus within the vascular endothelium (Wakefield *et al.* 1995; Wakefield *et al.* 1997), but this hypothesis merits further study. Detection of organisms in IBD patients does not of course necessarily establish them as primary aetiological factors. The delicate balance between luminal microbial constituents and protective mucosal forces can be disturbed by genetically determined immunoregulatory abnormalities and environmental triggers, leading to chronic, relapsing intestinal inflammation (Sartor *et al.* 1996b). Furthermore, it seems unlikely that a specific infection could alone explain the chronically relapsing and remitting natural history of IBD. More probably, one or more infective agents, or their products, trigger IBD, as a hit and run event, by stimulation of an inappropriately hyper-responsive mucosal immune system (MacPherson *et al.* 1996).

Mucus. The oligosaccharide side chains of mucus glycoproteins are shorter in patients with UC than controls, and show altered binding patterns with monoclonal antibodies and lectins (Stevens *et al.* 1992; Podolsky & Fournier, 1988; Rhodes *et al.* 1988). Compared with healthy subjects, the different biochemical features of the mucin obtained from whole gut lavage fluid from IBD patients appear to reflect mucosal pathological changes associated with the disease (Saitoh *et al.* 1996). MUC2 is the predominant mucin in the human colon responsible for the protective mucus layer. MUC2 precursor biosynthesis and total MUC2 levels were significantly decreased in UC patients with active inflammation compared to controls, and returned to control values during remission of the inflammation (Tytgat *et al.* 1996). Colonic mucin is heavily sulphated and it has been shown that enzymatic desulphation by faecal bacterial sulphatases greatly increases its susceptibility to degradation by faecal glycosidases. The increased faecal mucin sulphatase activity in UC could be the result of greater intraluminal substrate (mucin) availability leading to bacterial enzyme induction, but would probably result in more rapid degradation of secreted mucin and represents a potential target for treatment (Tsai *et al.* 1995). The striking increase in mucin synthesis that results when butyrate is added to standard nutrient medium in colonic biopsy cultures, suggests that this may be an important mechanism affecting the rate of mucin synthesis *in vivo* and may also explain the therapeutic effect of butyrate in colitis (Finnie *et al.* 1995). Nicotine, and possibly smoking, may affect colitis by an action on mucosal eicosanoids and on adherent surface mucus increased secretion in the rectum and large bowel (Cohen & Hanauer, 1996; Zijlstra *et al.* 1994). The marked stimulation of mucin synthesis by corticosteroids suggests that this may account, at least in part, for their therapeutic effect in UC (Finnie *et al.* 1996).

Mucosal permeability. Blood lactulose concentration in patients with IBD were found significantly increased over the controls, representing an increase of intestinal permeability in patients with IBD. This increase of blood lactulose may represent a disturbance of the intestinal barrier function due to mucosal changes such as erosion, ulceration, and oedema

(Hollander, 1992). Patients with CD have increased mucosal permeability to small molecular weight probes such as Cr-EDTA (Bjarnason & Peters, 1987). Lindberg et al using a group of monozygotic twin pairs concordant and discordant for CD were found no differences in the absorption of polyethylene glycols between the study groups (Lindberg *et al.* 1995). These results give no support to the hypothesis of a genetically determined intestinal leakiness in CD. In another study the intestinal permeability and the immune response to enteric bacterial antigens in patients with inactive CD were significantly increased over those in the controls as well as in patients with inactive UC (Oriishi *et al.* 1995). Whether increased gut permeability in CD is a primary abnormality predisposing to increased mucosal access of luminal pathogenic factors, or is simply a consequence of mucosal damage induced by other mechanisms is not yet resolved.

Colonocyte energy metabolism. Over the past decade it has become evident that normal colonic epithelium depends largely on short chain fatty acids (SCFA) derived from luminal bacterial metabolism for its energy supply. The principal SCFAs, acetate, propionate, and butyrate, are formed by bacterial fermentation of unabsorbed carbohydrates (Cummings & MacFarlane, 1991) and tend to be present in colonic contents in relatively constant concentrations and proportions (Mortensen *et al.* 1991). SCFAs are absorbed from the colonic lumen (Ruppin *et al.* 1980), and once inside the colonocytes, the cellular oxidation of SCFAs is considered to be the major energy source for the epithelial cells (Roediger, 1980b). In UC, colonocytes appear less able to utilise butyrate as an energy source and low luminal SCAF levels in severe UC may exacerbate this defect (Roediger, 1980a). SCFA are potentially valuable as a topical therapy for distal UC. Treatment with SCFA enemas were found to have therapeutic benefit in patients with distal UC (Breuer *et al.* 1997). The mechanism of action is unknown, but evidence that butyrate oxidation is impaired in colonocytes isolated from patients with UC has led to the hypothesis that failure of fatty acid oxidation in UC is an expression of an energy deficiency disease of the colonic mucosa (Breuer *et al.* 1991). Isolated human colonocytes

were found capable of utilising each of the three major SCFAs. Kinetic studies on colonocyte metabolism have shown a specific role of butyrate as an energy source for the colonic mucosa in both health and UC without support for either a pathogenic role for defective metabolism of butyrate in UC (Clausen & Mortensen, 1995) or to define whether abnormal colonic epithelial SCFA utilisation in UC is a primary or secondary phenomenon.

Cigarette smoking. UC is primarily a disease of non-smokers (Harries *et al.* 1982). The risk of developing disease after cessation of smoking is 'dose-related' by pack-years, and former smokers may be at higher risk than non smokers. In marked contrast CD is less favourable in smokers than in non-smokers (Breuerkatschinkski *et al.* 1996) and patients with CD should be dissuaded from smoking (Lindberg *et al.* 1992). Nicotine in chewing gum and transdermal patches has been shown to improve clinical symptoms and endoscopic appearance in patients with active UC, although no maintenance benefit has been seen (Cohen & Hanauer, 1996; Pullan *et al.* 1994), while topical administration of nicotine may be useful treatment for distal UC (Green *et al.* 1997). Proposed mechanisms for the 'protective' effect of nicotine in ulcerative colitis include modulation of the humoral and cellular immune response, increase mucus (Finnie *et al.* 1996), decreased mucosal eicosanoid levels (Zijlstra *et al.* 1994), alteration of rectal mucosal blood flow, decreased intestinal permeability, release of endogenous glucocorticoids (Cope & Heatley, 1992). Nicotine was found to cause a significant inhibition of IL-2 and TNF-alpha production (Vandijk *et al.* 1995; Madretsma *et al.* 1996), and an inhibitory effect on Th2 cell function as measured by inhibition of IL-10 production (Madretsma *et al.* 1996). The beneficial effects of smoking and nicotine in CD could be attributed to this inhibition, however, why UC and CD have such different relationship to smoking is at present a mystery.

Drugs NSAIDs have been associated with initiation and relapse of IBD, changes in eicosanoid metabolism and gut permeability being two possible mechanisms (Rampton,

1987). Although the confounding effects of concurrent smoking are difficult to exclude altogether (Katschinski *et al.* 1993), there appears to be an increased risk of CD in users of the oral contraceptive pill (Lesko *et al.* 1985; Boyko *et al.* 1994), perhaps as a result of the prothrombotic effects of the drug. This increased risk for IBD seem to be due to the thromboembolic disorders and intestinal ischaemia caused by the oral contraceptives, which trigger a chain of events that culminate in clinical IBD (Koutroubakis *et al.* 1996). Anecdotal reports relate antibiotic usage to relapse of IBD, but this could be due to changes in colonic bacterial flora or colonic ischaemia.

Psychological factors Although most physicians and some patients consider psychosocial factors important in aggravating already existing IBD and patients often comment that emotional stress triggers relapse, formal studies have not shown any influence of stressful life events on the natural history of IBD (North *et al.* 1991), while studies have shown that there are substantial personality differences between patients with CD and UC (Barrett *et al.* 1996). Stress is associated with alterations in both humoral and cellular immune mechanisms in humans and in experimental animals and mind-gut interactions affect salivation, gastric secretion, gastric motility and colonic motility, as well as numerous other gastrointestinal functions. While psychosocial factors may not initiate inflammation in IBD, it is possible that they lead to alterations in the immune response and thereby alter disease activity (Bayless, 1995). In the 1970s and early 1980s, several studies accurately identified that the cause of IBD was not psychosomatic, but rather that patients with these diseases had higher levels of stress and anxiety as a result of the diagnosis of a chronic disease (Talal & Drossman, 1995).

1. 2. 2 Pathogenesis of IBD

Although the primary aetiological agents in IBD is still elusive, we now know that disease activation involves an early increase in expression of surface adhesion molecules on

vascular endothelial cells, with consequent diapedesis and activation of circulating leukocytes in gut mucosa (Schuermann *et al.* 1993; Koizumi *et al.* 1992), and an increased synthesis and release of a wide range of inflammatory mediators and cytokines likely to contribute to the clinicopathological features of IBD. The sources of these mediators include not only resident and newly recruited mucosal inflammatory cells such as neutrophils, macrophages and mast cells, but also platelets, colonic epihelial cells, vascular endothelial cells, fibroblasts, smooth muscle cells and enteric neurons (MacDonald & Murch, 1994).

Prostaglandins. Eicosanoids seem to be implicated in the pathophysiology of IBD (Yang, 1996), as concentrations of all eicosanoids and soluble Fc gamma receptors (sFc gamma RIIIb) were found significantly increased in gut lavage fluid from patients with IBD (Hommes *et al.* 1996). Efforts have been made to inhibit the production of potentially damaging eicosanoids. Diets with fish oil changes intestinal eicosanoid synthesis, however, its utility in patients with active or inactive inflammatory disease is controversial (Casellas & Guarner, 1996). Historically, the prostaglandins (PG) were the first eicosanoids to be studied in IBD, their increased production in active disease being shown by a variety of methods including *in vitro* culture of colorectal biopsies and *in vivo* rectal dialyses (Rampton & Hawkey, 1984; Lauritsen *et al.* 1989; Baumeister *et al.* 1996). Luminal release of PGE_2 was found significantly higher in patients with CD than in control subjects. Furthermore, there was a modest, but significant increase in luminal PGE_2 in first-degree relatives and suggested that this increased synthesis of prostaglandins may constitute a response to altered genetic mucosal characteristics (Ahrenstedt *et al.* 1994). The increased PGE_2 production found in the inflamed mucosa in active UC may be caused by a fraction of activated eosinophils and macrophages (Raab *et al.* 1995). Theoretically at least, increased prostaglandin synthesis in IBD could account for diarrhoea, by inducing mucosal secretion of water and electrolytes and through effects on intestinal motility (Schmitz *et al.* 1996), it could also have a mucoprotective effect by

increasing mucus release, altering mucosal blood flow and suppressing immune and inflammatory cell function (Rampton & Hawkey, 1984; Lauritsen *et al.* 1989; Rampton & Collins, 1993). 5-aminosalysilate (5-ASA), which is successfully used in the treatment of IBD, did not affect the PGE_2 production in colonic mucosa, but decreased the LTB_4 synthesis in a dose related fashion (Schmidt *et al.* 1996). In addition the adverse effects of NSAIDs in IBD could be related to inhibition of prostaglandin synthesis (Davies, 1995).

Leukotrienes Leukotrienes are involved in the inflammatory processes of intestinal lesions. Many studies, both *in vitro* and *in vivo*, have shown increased mucosal production of leukotrienes in patients with active IBD (Rampton & Hawkey, 1984; Lauritsen *et al.* 1989; Cole *et al.* 1996; Baumeister *et al.* 1996). Colonic biopsies from untreated patients with active IBD showed a significant increase in LTB_4 synthesis compared with healthy controls. However, in patients receiving steroids, sulphasalazine or 5-aminosalicylic acid, LTB_4 was markedly decreased. Drugs effective in the treatment of these diseases may exert their pharmacological action by normalising these pathological findings (Schmidt *et al.* 1995). These compounds are likely to contribute to mucosal inflammation principally by their effects on the recruitment and activation of neutrophils. Inflamed colonic mucosa was found to release more neutrophil movement inducing bioactivity than uninflamed mucosa, and to have greater LTB_4 dependent activity (Cole *et al.* 1996). Selective leukotriene inhibitors and receptor antagonists are currently under evaluation in the treatment of IBD (Roberts *et al.* 1997) and Zileuton, a 5-lipoxygenase inhibitor, was found beneficial in IBD (Hawkey *et al.* 1997). This effect may be related to an increased and maintained production of PGE_2 together with inhibition of LTB_4 synthesis (Bertran *et al.* 1996). Importantly, the reported experience to date has shown that the leukotriene inhibitors do not have the same side-effects as the current therapies, promising that both safe and effective treatment may be derived from this approach (Harris *et al.* 1997).

Thromboxanes. Thromboxanes are produced in excess not only in inflamed mucosa, but also in CD by uninflamed bowel and by isolated intestinal and peripheral blood mononuclear cells (Rampton & Collins, 1993). Their pro-inflammatory effects are both direct (diapedesis and activation of neutrophils, mucosal ulceration, reduction of suppressor T-cell activity) and indirect (vasoconstriction, platelet activation) (Rampton & Collins, 1993). Indeed, multifocal microvascular infraction has been proposed as an early pathogenic factor in CD (Wakefield *et al.* 1989), in which increased platelet aggregation and activation is common (Collins *et al.* 1994), and it is possible that enhanced thromboxane production contributes to these phenomena. Low dose aspirin will selectively inhibit a proportion of rectal thromboxane and may have prophylactic therapeutic potential in IBD (Cole *et al.* 1994). Selective thromboxane synthesis inhibitors and receptor antagonists such as picotamide and ridogrel are available for use in man and each has been found promising in studies in IBD (Casellas *et al.* 1995; Collins *et al.* 1996). The classical therapeutic drugs used in IBD, namely corticosteroids and derivatives of sulfasalazine, have significant effects on eicosanoid synthesis, providing evidence for their role in IBD. In the future, specific subgroups of patients with IBD may benefit from new eicosanoid inhibitors (Casellas & Guarner, 1996).

Platelet activating factor Recent data shows increased synthesis and content of platelet activating factor (PAF) in the colorectal mucosa of patients with UC and correlated to local injury and inflammation (Guimbaud *et al.* 1995; Wardle *et al.* 1996). In contrast, other studies did not find high concentrations of PAF in the rectal mucosa of patients with active UC compared with patients in remission or controls, and they suggested that PAF has not an important role as a mediator of inflammation of UC (Almer *et al.* 1996). PAF stimulates mucosal chloride secretion and inhibits sodium chloride absorption (Bern *et al.* 1989), so it could contribute to the production of diarrhoea in UC. The PAF receptor antagonist lexipafant (BB-882) shows efficacy in treating inflammation in an animal model of acute colitis as evidenced by a dose-dependent fall in macroscopic mucosal

damage, neutrophil infiltration and reduced generation of inflammatory mediators (Meenan *et al.* 1996). However, there have been no published reports of the effects of PAF antagonists in human IBD, and the pathogenic importance of this mediator remain uncertain.

Biogenic amines Patients with UC have increased rectal mucosal release of histamine (Rampton *et al.* 1980). In recent studies, histamine content and secretion were found to be significantly increased particularly in affected mucosa of CD and UC than in unaffected tissue or in healthy controls. These findings give strong evidence that mast cell mediators like histamine play a role in the pathogenesis of these diseases (Raithel *et al.* 1995). This increased histamine secretion could contribute to vasodilatation, increased vascular permeability, sensory nerve activation, smooth muscle contraction and mucosal fluid and electrolyte secretion in intestinal inflammation.

Kinins Increased secretion of kinins occurs in animal models of colitis but has not been reported in IBD (Lauritsen *et al.* 1989). Kinins have many biological effects, including vasodilatation, increased vascular permeability, mobilisation of blood cells and mucosal fluid secretion, that appear to be mediated by eicosanoids. The kallikrein-kinin (K-K) system has suggested to be directly involved in the pathogenesis of the acute phase of experimental acute intestinal inflammation (Stadnicki *et al.* 1996). Activation of the K-K system plays an important role in experimental models of inflammation, suggesting that activation of this system may play a role in the pathogenesis of IBD and suggests that this pathway is one determinant of genetic susceptibility to granulomatous enterocolitis and systemic complications (Delacadena *et al.* 1991; Sartor *et al.* 1996a).

Proteases Studies of the tissue levels of phagocyte-derived proteases have given conflicting results. Acid proteases derived from the secondary granules of neutrophils are present in increased amounts in IBD mucosa (Kane & Vincenti, 1979; O'Morain *et al.*

1983), but levels of the constituents of primary granules (elastase, cathepsin D, chymotrypsin) are increased (Seitz *et al.* 1995), normal or decreased (Kane & Vincenti, 1979). In a rabbit model of chronic colitis, collagenase was found to be associated with the acute phase of ulcer formation, whereas stromelysin and gelatinase are predominant during healing (Anthony *et al.* 1994). Gastrointestinal epithelium contains a powerful protease inhibitor called pancreatic secretory trypsin inhibitor (PSTI). Reduction in mucosal PSTI in UC patients may be related with changes in mucus structure suggestive of increased proteolysis (Playford *et al.* 1995). While direct measurements of tissue concentrations of proteases can be complicated by autoactivation, it is conceivable that enzyme activity contributes to mucosal damage in IBD by degradation of elastin, collagen and other connective tissue proteins. The effects of protease inhibitors in human IBD has not been assessed.

Reactive oxygen species The production of reactive oxygen species (ROS) was found increased in inflamed tissue, peripheral blood monocytes and isolated intestinal macrophages from patients with IBD (Simmonds & Rampton, 1993; Grisham, 1994). Levels of endogenous antioxidants, such as superoxide dismutase, metallothionein and glutathione peroxidase, were found reduced (Lihbrody *et al.* 1996). Activation of granulocytes and monocytes/macrophages at the site of inflammation increases the production of reactive oxygen metabolites both in animal models of experimental intestinal injury, and in IBD (Gross *et al.* 1994). Evidence of ROS-mediated tissue damage comes from detection of lipid peroxidation in mucosal biopsies in active UC (Anhfelt-Ronne *et al.* 1990). ROS have a wide range of pro-inflammatory actions which are likely to contribute to the pathogenesis of IBD, a conclusion made more credible by preliminary reports of the beneficial effects of antioxidant agent such as superoxide dismutase (Emerit *et al.* 1989), allopurinol and dimethylsulphoxide (Salim, 1992), and tirilazad mesylate (Yue *et al.* 1996) in IBD. Some of the anti-inflammatory actions of aminosalicylates have been ascribed to their capability to scavenge superoxide radicals directly or to inhibit its

production in stimulated neutrophils (Allgayer *et al.* 1994). Oxidation and inhibition of essential protein function by inflammatory cells is a potential mechanism of tissue injury that may contribute to the pathogenesis of the disease and supports the exploration of compounds with antioxidant activity as new therapies for IBD (McKenzie *et al.* 1996). If confirmed, such treatment could reduce the risk of colon cancer in patients with UC by preventing oxidative DNA damage (Simmonds & Rampton, 1993).

Complement. Immunohistochemical studies have detected deposits of terminal complement complex in the muscularis mucosa and submucosal blood vessels in inflamed gut wall in IBD (Halstensen *et al.* 1989) and on the brush border of colonic epithelial cells from UC patients (Halstensen *et al.* 1990). Increased secretion of C3 and C4 has been reported into the lumen of uninvolved jejunum in patients with CD apparently confined to the terminal ileum (Ahrenstedt *et al.* 1990) and C3b on the brush border of colonocytes in patients with active UC (Halstensen *et al.* 1990). Deposition of the C3 fragments occurs in inflamed colonic mucosa of diverse aetiologies, including UC, but to define a role of the deposition in the development of mucosal injury in UC awaits further study (Ueki *et al.* 1996). As well as participating in neutrophil-endothelial cell interactions, complement components may contribute to intestinal inflammation by stimulating release of histamine, eicosanoids, PAF and IL-1 (Lauritsen *et al.* 1989; Halstensen *et al.* 1990).

Neuropeptides: Regulatory neuropeptides are widely distributed in the gastrointestinal tract, where they play an important role in motility, secretion, immune and inflammatory responses (Sanahan & Anton, 1988). Recent studies suggest that substance P (SP), and vasoactive intestinal polypeptide (VIP) play a role in inflammatory processes of the bowel. An increase in the density of SP-immunoreactive nerve fibres has been found in inflamed ileal pouch mucosa of clinically asymptomatic pouchitis patients (Keranen *et al.* 1996), while decreased tissue levels of VIP reported in chronic colonic inflammation could be due to diminished gene expression of VIP observed in UC (Schultebockholt *et al.* 1995).

In another study, in active UC, VIP- and SP-nerves decreased in severe inflammatory lesions and VIP-nerves were almost absent particularly around crypt abscesses. In the uninvolved mucosa of UC, they did not change their distribution. In CD, the distribution abnormality of both nerves resembled that of UC (Kimura *et al.* 1994). The expression of receptors for somatostatin (SOM) in veins of inflamed intestines suggests an active involvement of this peptide in the pathophysiology of IBD (Reubi *et al.* 1994). The content of immunoreactive-SOM was decreased in UC patients, especially in those with persistent disease activity, while the levels of immunoreactive-SP, beta-endorphin (BE), and thyrotropin-releasing hormone (TRH) were increased in such patients (Yamamoto *et al.* 1996). The pro-inflammatory effects of SP suggest that it may play a role in the pathogenesis of IBD and the report that lidocaine enemas are efficacious in proctitis (Bjorck *et al.* 1989) is of interest. However, topical anaesthetics may not act solely through interference with enteric nerve function since they have recently been shown to inhibit neutrophil ROS, leukotriene B_4 and IL-1 production directly *in vivo* (Sinclair *et al.* 1993).

Cytokines. Cytokines are a large family of structurally diverse proteins of 8-30 kD, which are produced by most nucleated cells, particularly but not exclusively immune cells. These proteins probably play a key role in intestinal inflammation via inducing and suppressing inflammatory process. In addition they have a role in the regulation of healing and repair (Dieleman *et al.* 1996) by activating effector cells (Pallone & Montelleone, 1996), by increasing their proliferation and providing chemotactic messages. Individual cytokines modulate the secretion not only of others, but also other mediators. Thus cytokines may be responsible for many of the symptoms in patients with IBD via their local and systematic effects or inducing the production of other mediators and initiating a cascade of effects (Fiocchi *et al.* 1994).

IL-1 has a large range of biological activities, including the induction of cytokines, chemokines, adhesion molecules, enzymes (cyclo-oxygenase, soluble phospholipases,

collagenases) and it is regarded now as a major pro-inflammatory cytokine (Dinarello, 1996). In active IBD, isolated peripheral blood mononuclear cells were found to produce increased levels of IL-1 compared to controls (Grottrupwolfers *et al.* 1996), but reverted to control levels in the inactive stages and they found significant correlation between the IL-1β production and the activity index of the diseases (Nakamura *et al.* 1992). IL-1β mRNA was found in colonic biopsies from patients with infectious and ischaemic colitis. These results suggest that production of IL-1β is not unique to active IBD but is also increased in intestinal inflammation (Isaacs *et al.* 1992). Organ cultures of involved IBD mucosa spontaneously produced increased amounts of IL-1β compared to normal mucosa (Reimund *et al.* 1996). The increased IL-1 production at the mucosal level in patients with active IBD is probably derived from the presence of increased number of cells capable of synthesising IL-1 in the inflamed intestinal mucosa (Grottrupwolfers *et al.* 1996). Macrophages have been suggested as the major source of IL-1 in IBD mucosa (Mahida *et al.* 1989). However, there may be other cell types than macrophages involved in IL-1 production. Finally, levels of IL-1ra were markedly increased in patients with IBD and an imbalance in the ratio of tissue IL-1 and interleukin-1 receptor antagonist (IL-1ra) levels in the intestinal mucosa of patients with IBD has been proposed (Kam *et al.* 1995).

IL-2, the first of a series of lymphocytotropic hormones to be recognised and completely characterised, is pivotal for the generation and regulation of the immune response (Smith, 1988). In the investigation of IL-2 in IBD conflicting data have been published (Radford-Smith & Jewell, 1996). Decreased or absent IL-2 production by blood mononuclear cells (Ebert *et al.* 1984), mucosal mononuclear cells or both (Fiocchi, 1989) has been reported in IBD. Although T lymphocytes are numerous in normal bowel mucosa and their number increases in CD and UC, abnormalities of IL-2 production and response have been reported in IBD (Ebert *et al.* 1984). In other studies, using ELISA, increased concentrations of IL-2 have been demonstrated in both plasma and endoscopic biopsies from bowel mucosa of patients with active IBD, or using polymerase chain reaction (PCR), presence of increased

mucosal T-cell IL-2 mRNA transcripts has been detected in IBD patients (Mullin *et al.* 1992). It has been suggested that the increased production of the soluble form of the IL-2 receptor (sIL-2R), observed in IBD, causes the insignificant change in IL-2 production trapping of the free IL-2 (Mueller *et al.* 1990). An explanation for these contradictory results may be that the IL-2 production varies with the degree or the type of the inflammation and the stage of the disease. IL-2 production by intestinal lamina propria mononuclear cells in an animal experimental model has been found decreased in an early stage (acute phase) and increased in a later stage (chronic phase) (Gurbindo *et al.* 1993). Recent studies of IL-2 deficient mice revealed that IL-2 has a key function in intestinal immune homeostasis and might play a crucial role in the pathogenesis of IBD (Ehrhardt *et al.* 1997).

Elevated levels of IL-6 in serum and bowel mucosa have been demonstrated in IBD. This is confirmed by the detection of IL-6 mRNA transcripts in actively inflamed tissues from IBD patients (Isaacs *et al.* 1992). IL-6 positive IBD specimens were also found positive for IL-1ß and it has been suggested that the expression of IL-6 may provide an additional mediator in IBD (Grottrupwolfers *et al.* 1996) that, in common with IL-1β, and other mediators leads to the inflammatory response characteristic of IBD, or IL-6 play a protective role by inducing hepatic acute phase proteins (Stevens *et al.* 1992). Organ cultures of involved IBD mucosa spontaneously produced increased amounts of IL-6 compared to normal mucosa (Reimund *et al.* 1996). A significant relationship between serum IL-6 antigen and platelet count has been reported in IBD (Hyams *et al.* 1993). It has been suggested that IL-6 may stimulate megacaryocyte activity and result in thrombocytosis in disease characterised by chronic inflammation (Hollen *et al.* 1991). Taken together these reports suggest that elevated serum IL-6 levels may be associated with elevated platelet counts frequently seen as part of the acute phase response in IBD patients.

Studies on TNF-α production have detected no significant differences in serum or mucosal specimens between IBD patients and normal controls (Greenfield *et al.* 1993), whereas organ

cultures of involved IBD mucosa were found to produce increased amounts of TNF-α compared to normal mucosa (Reimund *et al.* 1996). Tissue levels of TNF-α mRNA were not found increased in IBD specimens using PCR amplification (Isaacs *et al.* 1992) and it has been suggested that the increase of IL-6 in IBD, which inversely regulates TNF-α production, may be responsible for the apparent decrease expression of TNF-α in intestinal inflammation (Isaacs *et al.* 1992). The systemic levels of TNF-α were not found to be elevated in an experimental model of chronic colitis compared to controls and it has been suggested that other pro-inflammatory mediators with biological properties parallel to those of TNF-α - as IL-1 - may be responsible for the systemic manifestations of chronic colitis (Mack *et al.* 1992). In another study the amount of TNF-α in the stools has been referred as a marker of intestinal inflammation (Braegger *et al.* 1992). TNF-α has a large range of biological activities, most similar with those of IL-1, including the induction of cytokines and enzymes that are involved in the generation of inflammatory mediators (Beutler & Cerami, 1995). In addition, TNF-α has been found to increase adhesion molecule expression on the cell membranes of monocytes and granulocytes and it has been suggested that this cytokine might be responsible for leukocytes recruitment into the bowel wall in IBD (Greenfield *et al.* 1993; Lo *et al.* 1989).

Although IFN-γ was originally defined as an agent with direct antiviral activity, now we know that the properties of this lymphokine include regulation of several aspects of the immune response (Boehm *et al.* 1997). The reports about IFN-γ production in IBD are contradictory (Radford-Smith & Jewell, 1996). Serum levels of IFN-γ have been found elevated in patients with active disease (Simon *et al.* 1983; Stalnikowicz *et al.* 1985) and increased spontaneous release of IFN-γ by cultured mucosal mononuclear cells from CD patients has been reported, whereas peripheral blood mononuclear cells release IFN-γ after stimulation (Fais *et al.* 1991). In other reports, the production of IFN-γ in peripheral blood mononuclear leukocytes of patients with IBD was found to be the same as that in controls (Nakamura *et al.* 1992), mitogen activation of peripheral blood mononuclear cells from IBD

patients showed normal or decreased levels of IFN-γ (Miura & Hiwatashi, 1985; Stalnikowicz *et al.* 1985; Mutchnick *et al.* 1988) and stimulated intestinal mononuclear cells from both CD and UC patients have been detected to produce decreased levels of IFN-γ as compared to the normal controls (Lieberman *et al.* 1988). However, in experimental models, inflamed colonic mucosa from Gαi2-deficient mice with colitis exhibited increase in IFN-γ and an important role for this lymphokine in IBD has been suggested (Hornquist *et al.* 1997).

The pathogenesis of IBD may be associated with changes in the production of T-cell-derived cytokines IL-4, IL-10, and IL-13 (Kucharzik *et al.* 1997). These T-cell derived cytokines have all been proposed as anti-inflammatory cytokines. For example IL-13 is a potent suppressor of cytokine and chemokine expression by activated monocytes and macrophages (Minty *et al.* 1993; McKenzie *et al.* 1993; Zurawski & De Vries, 1994; De Waal Malefyt *et al.* 1993), and endothelial cells (Marfaing-Koka *et al.* 1995). In addition IL-13 induces the production of IL-1 receptor antagonist (Muzio *et al.* 1994) and modulates the expression of cell surface proteins such as class II MHC antigens (De Waal Malefyt *et al.* 1993). Similar biological activities are also displayed by IL-4, in addition both cytokines are potent stimulators of B-cell IgE production, while IL-4 alone has an effect on human T-cells (Zurawski & De Vries, 1994). Interleukin-10 is produced by a variety of cells including activated human T-cells, and it is also a potent suppressor of cytokine and chemokine generation by activated monocytes/macrophages (Moore *et al.* 1993; Hsu *et al.* 1990; Vieira *et al.* 1991; de Waal Malfyt *et al.* 1991; Fiorentino *et al.* 1991) and polymorphonuclear cells (Kasama *et al.* 1994). IL-4 mRNA expression is decreased in intestinal tissue from CD patients, while IL-10 mRNA expression is decreased in majority of UC patients, suggesting different immunopathogenesis of the two diseases (Nielsen *et al.* 1996). IL-10 was found able to down-regulate all proinflammatory cytokines in active IBD as well as in controls (Kucharzik *et al.* 1996) and IL-10 deficient mice were found to develop chronic enterocolitis (Kahn *et al.* 1993; Rennick *et al.* 1997). Activated monocytes with increased expression of proinflammatory cytokines play a major role in IBD. Immunoregulatory

cytokines such as IL-4 and IL-10 can effectively suppress the proinflammatory response of activated monocytes (Kucharzik *et al.* 1997). With regard to IL-13 and IL-4, there was no significant suppression of TNF-α and IL-6 in patients with active IBD (Kucharzik *et al.* 1996). By contrast, IL-10 was able to down-regulate all proinflammatory cytokines in active IBD as well as in controls (Kucharzik *et al.* 1996; Murata *et al.* 1995). *In vivo* topical application of IL-10 induces down-regulation of proinflammatory cytokine secretion both systemically and locally (Schreiber *et al.* 1995). The results about the IL-13 production in IBD patients are contradictory so far (Radford-Smith & Jewell, 1996). The inhibitory effect of IL-13 on TNF-α and IL-6 production in differentiated macrophages was diminished in IBD patients and the anti-inflammatory activity of IL-13 was found partially reduced in patients with active IBD (Kucharzik *et al.* 1996), while IL-13 has been found to inhibit nitric oxide production in activated colonic epithelial cells (Kolios *et al.* 1997).

The aetiology of IBD appears to be immunologically mediated and cytokines with their inflammatory and regulatory properties appear to play a role in pathogenesis of UC and CD. These molecules have simultaneous protective effects that are important in the resistance to infectious agents and possibly in the healing phase of inflammation and the relative balance of the inflammatory and protective properties of the cytokine network may determine the chronicity of inflammation and the tendency of reactivation of these diseases.

Conclusions. The aetiology of IBD remains obscure. It seems most likely that environmental factors, for example dietary or microbial, trigger an inappropriate mucosal inflammatory response in individuals having genetically abnormal immune system, gut permeability, mucus and/or colonocyte metabolism. Although the pathogenesis of IBD is gradually being elucidated, we remain ignorant of the mechanisms underlying the

Table 1. Soluble inflammatory mediators in IBD

Group	Members	Effect
Eicosanoids	Prostaglandins, Leukotrienes Thromboxanes	Regulation of electrolyte secretion, diarrhoea, transmigration and activation of neutrophils, platelet activation, vasoconstriction
Platelet activating factor		Regulation of sodium chloride secretion, diarrhoea
Biogenic amines	Histamine	Vasodilatation, vascular permeability, smooth muscle contraction
Kinins	Kallikrein-kinin	Intestinal secretion, activation of prostaglandin formation
Proteases	Elastase, cathepsin, collagenase, chymotrypsin	Mucosal damage, degradation of connective tissue proteins
Reactive oxygen species	Superoxide anion, hydrogen peroxide, perhydroxyl radical	Tissue damage, cellular toxicity, oxidative DNA damage, mutation, development of neoplastic disorders
Complement components	C3, C3b, C4	Stimulation of histamine, eicosanoids, PAF, IL-1 formation
Cytokines	IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-13, TNF-α, IFN-γ	Activation of effector cells, cell proliferation and communication, induction and suppression of inflammatory responses
Chemokines	IL-8, MCP-1, RANTES, eotaxin, MIP-1 α, MIP-1β	Transmigration and activation of specific leukocyte populations
Nitric oxide/Nitric oxide synthases	iNOS, cNOS	Cytotoxicity, vasodilatation, peroxynitrate formation, protective effects
Neuropeptides	Substance P, somatostatin, vasoactive intestinal polypeptide	Regulation of motility and secretion, protective effects

(See text for references)

chronicity of mucosal inflammation and relapse of quiescent disease in patients with IBD. For example, is chronicity due to persistence of an exogenous stimulus of inflammation, or to genetically defective down-regulation of the immune response (Podolsky, 1991)? Answers to these questions are a priority if we are to make major strides in our understanding of the pathogenesis of IBD, and in its treatment.

1. 3 CHEMOKINES

The discovery of IL-8 in 1987 revealed the existence of a superfamily of inducible, proinflammatory cytokines (Baggiolini *et al.* 1989; Rollins, 1997). The members of this family are small, mostly 8-14 kD, heparin-binding polypeptides, that are involved in attracting effector cells to the site of inflammation and also participate in activation of specific leukocytes (Lindley *et al.* 1993). The production of chemokines is not limited to immune cells, but a wide spectrum of tissue cells e.g. fibroblasts, endothelial cells and mesangial cells, expresses and secretes these cytokines upon appropriate stimulation, e.g. IL-1, TNF, IFN-γ, LPS (Baggiolini *et al.* 1994). These proteins are structurally related by possessing four conserved cysteine residues that form two disulphide bonds and establish the tertiary structure of the protein. The key feature of chemokines is their ability to attract subsets of leukocytes in a relatively specific manner and they are divided into sub-families based on the arrangement of the conserved cystein residues in the molecule (Oppenheim *et al.* 1991). Four groups of chemokines have now been defined.

The C-X-C or α sub-family (see Table 2), with IL-8 the prominent member, has an intervening aminoacid residue between the first two cysteine residues and attract and activate neutrophils (Baggiolini *et al.* 1989). The C-C (see Table 3) or β sub-family in which the first two cysteine residues are adjacent, which include monocyte chemotactic protein-1 (MCP-1) and Regulated on Activation, Normal T cell Expressed and Secreted

Table 2. CXC Chemokines

Name	Target cells
ERL	
IL-8	Neutrophils, lymphocytes, basophils, angiogenesis (?endothelial cells)
GRO-α(MGSA)	Neutrophils, melanoma cells, (?endothelial cells)
GRO-β(MIP-2α)	Neutrophils, (?endothelial cells)
GRO-γ(MIP-2β)	Neutrophils, (?endothelial cells)
ENA 78	Neutrophils
GDP-2	Neutrophils
Platelet basic protein	
CTP-III	Fibroblasts
β-Thromboglobulin	Fibroblasts
NAP-2	Neutrophils, basophils
non-ERL	
Platelet factor 4	Fibroblasts, endothelial cells
IP-10	Activated T lymphocytes, TIL, ?endothelial cells, NK cells
MIG	Activated T lymphocytes
SDF-1α	T lymphocytes, $CD34^+$ progenitors, ?B lymphocytes
SDF-1β	?

(Rollins, 1997)

Table 3. CC Chemokines

Name	Target cells
MCP-1	Monocytes, memory T lymphocytes, basophils, NK cells, hematopoietic progenitors, ?dendritic cells
MCP-2	Monocytes, memory and naive T lymphocytes, basophils, NK cells, eosinophils
MCP-3	Monocytes, memory T lymphocytes, basophils, NK cells, dendritic cells, eosinophils
MCP-4	Monocytes, T lymphocytes, eosinophils
MCP-5	Monocytes, T lymphocytes, eosinophils
MIP-1α	Monocytes, T lymphocytes, basophils, NK cells, dendritic cells, eosinophils, hematopoietic progenitors
MIP-1β	Monocytes, T lymphocytes, NK cells, dendritic cells, hematopoietic progenitors
MIP-1γ (mouse only)	Resting and activated T lymphocytes
RANTES	Memory T lymphocytes, basophils, NK cells, eosinophils dendritic cells
Eotaxin	Eosinophils
I309	Monocytes
HCC-1	Monocytes, hematopoietic progenitors
TARC	T lymphocytes
C10 (mouse only)	?
CCF 18 (mouse only)	T lymphocytes, hematopoietic progenitors
MIP-3α/LARC	?
MIP-3β	?

(Rollins, 1997)

(RANTES)) and preferentially act on mononuclear cells. MCP-1 is a potent chemoattractant for monocytes/macrophages (Leonard & Yoshimura, 1990), for $CD4^+$ and $CD8^+$ T lymphocytes (Loetscher *et al.* 1994; Taub *et al.* 1995), and for tumour-associated macrophages (Mazzucchelli *et al.* 1996b). In addition to monocytes, RANTES is a selective chemotactic agent for CD45RO/$CD4^+$ T cells (memory T cells) (Schall *et al.* 1990; Turner *et al.* 1995) and eosinophils (Kameyoshi *et al.* 1992). The discovery of lymphotactin (Kelner *et al.* 1994) has suggested the existence of a third group named C or γ subfamily which lacks two (the first and third) of the four conserved cysteine residues. Lymphotactin is a chemotactic cytokine for lymphocytes, but not neutrophils and monocytes and it is produced abundantly by $CD8^+$ T cells (Kennedy *et al.* 1995). Finally, the recently defined CX_3C chemokine (also referred to as "fractalkine" or "neurotactin"), that is an integral membrane protein with a chemokine domain in its N-terminus (Bazan *et al.* 1997), has established a new chemokine group. This subfamily differs from the other chemokines by the presence of three amino-acids intervening between the first two cysteins (Rollins, 1997).

The C-X-C subfamily can be further divided into proteins which contain or lack an ELR motif. This motif consists of three aminoacids Glu-Leu-Arg that precede the first cysteine residue in the molecule near the amno terminus and it plays a crucial role in receptor-ligand interactions on neutrophils. Chemokines with the ELR motif act primarily on neutrophils as chemoattractants and activators, inducing neutrophil degranulation. On the other hand the C-X-C chemokines, which lack the ELR domain have no neutrophil chemotactic activity. In addition, the ELR containing CXC chemokines (IL-8, Groα, Groβ, Groγ, ENA-78 and NAP) are angiogenic agents, while non ELR expressing chemokines (MIG, IP10 and PF-4) are angiostatic (Strieter *et al.* 1995). The chemokine subfamilies have a distinguished chromosomal location of their genes. C-X-C chemokines are localised on human chromosome 4 (q12-q21), while the C-C chemokines are clustered on

human chromosome 17 (Donlon *et al.* 1990). The gene encoding the C chemokine, lymphotactin is located on human chromosome 1 (Kennedy *et al.* 1995).

A conceptual leap in the field of cell migration has been the realisation that extravascular leukocyte accumulation is a multi-step process and requires a series of co-ordinated signals, including the expression and activation of cellular adhesion molecules as well as the generation of chemotactic gradients by the cells of the extravascular compartment (Huber *et al.* 1991; Adams & Shaw, 1994). Chemokines participate at several levels of the multi-step process that regulates leukocyte migration into injured tissue. This process is defined by four sequential steps: rolling, triggering, adhesion and migration (Adams & Shaw, 1994). In an initial step, leukocytes become attached to the endothelial layer but are still allowed to roll in a slower flow along the vessel wall. Rolling is mediated by selectins, which are expressed in both endothelium and leukocytes. However, without a further triggering factor leukocytes can still disengage from the vessel wall (Springer, 1994). Chemokines are important to enhance integrin adhesiveness, and to promotes strong adhesion (Vaddi & Newton, 1994). In the next step of extravasation, stimulated leukocytes spread and firmly attached to the endothelial layer through the interaction of integrins on the leukocytes with adhesion molecules on the endothelial cells (Adams & Shaw, 1994). IL-8 is an important example of a neutrophil triggering factor (Rot, 1992), while both RANTES and MIP-1β can activate the T cell integrins, VLA-4 (Very Late Antigen 4) and LFA-1 (Leucocyte Function antigen-1) (Gilat *et al.* 1994). After strong adhesion to the endothelium, leukocytes migrate into the underlying tissue along a chemotactic gradient. Migration of certain subsets of leukocytes into tissue depends on the type of integrin expressed on the leukocytes and on the interaction with a specific endothelial adhesion molecule, of which the expression depends on the type of endothelium and on the stimulating chemokine. This complex mechanism helps to migrate the correct leukocyte to a specific site (Kubes & Wallace, 1995).

Chemokines mediate their activities by binding to an array of shared and specific receptors on blood leukocytes (Schall, 1994; Sozzani *et al.* 1995) that belong to the family of heterotrimeric G protein-coupled receptors, which process seven transmembrane domains. To date four C-X-C chemokine receptors (CXCR-1 to 4) and eight C-C chemokine receptors (CCR-1 to 8) have been cloned and expressed (Power & Wells, 1996). CXCR-1 previously known as IL-8RA or IL-8 receptor type I is specific for IL-8, while CXCR-2 (IL-8RB or IL-8 receptor type II) also binds other C-X-C chemokines, including melanocyte growth stimulating activity/*gro* (MGSA/*gro*), epithelial-derived neutrophil attractant-78 (ENA-78) and neutrophil activating protein-2 (NAP-2) with high affinity (Holmes *et al.* 1991; Murphy & Tiffany, 1991). CXCR-3, also known as the IP-10/Mig receptor is a highly expressed by IL-2-activated T lymphocytes, B lymphocytes, monocytes and granulocytes, and binds IP-10 and Mig, but not PF4. This receptor does not bind the C-X-C chemokines that contain the ELR domain (Loetscher *et al.* 1996). CXCR-4 receptor also known as fusin is a necessary co-factor for entry of T cell tropic HIV viruses into $CD4^+$ cells (Feng *et al.* 1996) and is the specific receptor for SDF-1. Receptor binding studies indicate the existence of multiple receptors for C-C chemokines, which differ mainly in their NH_2-terminal part that might explain the different specificity of the C-C chemokines for particular receptor interactions.

An additional factor which may enhance this specificity may be the cellular distribution of the receptors (Sozzani *et al.* 1995). CCR-1 has been cloned, and binds MIP-1α, RANTES, MCP-3 with high affinity and MIP-1β and MCP-1 with lower affinity (Neote *et al.* 1993). CCR-2, which is specific for MCP-1, MCP-2, MCP-3 and MCP-4, has two forms (type A and B) that differ in their alternatively spliced carboxyl terminus and are probably spliced variants of a single gene (Charo *et al.* 1994). CCR-3 is a high affinity receptor for eotaxin, RANTES and MCP-3 (Kitaura *et al.* 1996; Daugherty *et al.* 1996). CCR-4 binds RANTES, MIP-1α, MCP-1, and has been shown to be expressed in T cells, B cells and monocytes (Power *et al.* 1995; Hoogewerf *et al.* 1996). CCR-5 is expressed in monocytes,

macrophages, and T cells and mediates the activities of RANTES, MIP-1α, and MIP-1β. Recently they found that this receptor is a co-receptor on $CD4^+$ target cells for infection with primary, monocyte-tropic HIV-1 viruses (Samson *et al.* 1996; Dragic *et al.* 1996). Furthermore, the Duffy blood group antigen, has been detected to be an erythrocyte chemokine receptor that can bind C-X-C and C-C chemokines (Neote *et al.* 1994). The Duffy antigen receptor for chemokines (DARC) present on human erythrocytes and endothelial cells may play a role in leucocyte transmigration by concentrating chemokines at the cell surface for presentation to target leukocytes and seem to be identical to the receptor for the invasion of the malarial parasite, *Plasmodium vivax* (Schall, 1994; Sozzani *et al.* 1995). An isoform of this receptor has also been localised on endothelial cells lining post-capillary venules in the kidney (Hadley *et al.* 1994).

Chemokines, together with adhesion molecules, cytokines and proteases are essential for the directional migration of leukocytes during normal and inflammatory processes (Proost *et al.* 1996). However, the degree of leucocyte specificity they possess, which contrasts with the non-specific action of the classical chemotactic factors such as C5a, platelet activating factor (PAF) and leukotriene B_4, may explain in part, the time-dependent accumulation of specific leucocyte populations during the course of acute and chronic inflammation (Adams & Shaw, 1994). Finally, chemokines are important not only in acute and chronic inflammation, but they have also been implicated in lymphocyte trafficking during T cell development and maturation, coagulation, haemopoiesis, angiogenesis, wound healing, autoimmune diseases, allergy, and malignancy (Proost *et al.* 1996).

1. 3. 1 The role of chemokines in IBD

The study of chemokine expression and production in IBD is still in its beginning with the majority of published data focusing on IL-8, during the course of this study. The movement of neutrophils into the colonic mucosa in UC is thought to be induced mainly by IL-8 and

leukotriene B-4 (Cole *et al.* 1996). Significantly higher levels of IL-8 have been found in macroscopically and histologically inflamed and non inflamed colonic mucosa from UC and CD patients than in controls (Daig *et al.* 1996) and in both the proximal and distal regions of the colonic mucosa of UC patients there was a more than 10-fold increase in IL-8 levels over that in control subjects (Izzo *et al.* 1992), an observation which might indicate more extensive disease than indicated by endoscopic and histological examination (Izzo *et al.* 1992). IL-8 mRNA has found in colonic biopsies from IBD patients, using PCR amplification and in situ hybridisation (Isaacs *et al.* 1992; Daig *et al.* 1996). Colonic epithelial cell lines have been reported as a source of IL-8 after stimulation by the pro-inflammatory cytokines IL-1α and TNF-α or lipopolysaccharide (Schuerer-Maly *et al.* 1994; Gross *et al.* 1995).

Freshly isolated human colon epithelial cells were found to express IL-8 (Jung *et al.* 1995) and it has been suggested that colonic epithelial cells may contribute to neutrophil extravasation and tissue infiltration in intestinal inflammation (Kelly *et al.* 1994). Furthermore, it has been found that increased levels of IL-8 in mucosal samples from UC patients was associated with increased levels of myeloperoxidase in corresponding regions of the colonic mucosa (Izzo *et al.* 1992). In situ hybridisation with IL-8 anti-sense RNA probes revealed strong and specific signals in the histologically affected mucosa in active IBD, but not in the mucosa of uninvolved bowel segments and in normal control mucosa. Tissue specimens from two patients with acute appendicitis displayed IL-8-expressing cells in the mucosa. These results suggest that IL-8 is not specific in the pathogenesis of IBD, but it is involved in the intestinal inflammation (Mazzucchelli *et al.* 1994). The role of IL-8 in IBD is unknown, though this cytokine is likely to induce the neutrophil chemotaxis and the accumulation of the large number of neutrophils in the intestinal inflamed mucosa in IBD (Brynskov *et al.* 1992; Kelly *et al.* 1994).

Peripheral blood monocytes are recruited from the circulation to the inflamed mucosa of IBD. Monocyte chemoattractant protein-1 (MCP-1) a chemokine with potent monocyte attracting and activating properties has been detected in large bowel mucosa from IBD patients. Freshly isolated human colon epithelial cells were found to express MCP-1 (Jung *et al.* 1995). MCP-1 transcripts were found generally increased in the intestinal mucosa of patients with IBD compared with controls, and MCP-1 gene expression in the mucosa was restricted to the lamina propria. Compared with controls, a significant increase of MCP-l-expressing cells was observed in tissue specimens from patients with IBD, in endothelial cells of venules, and in cells present in the lumen of intestinal vessels (Mazzucchelli *et al.* 1996a). In another study they found MCP-1 mRNA expression and MCP-1 protein in macrophages, some of which had been recently recruited from the as well as in smooth muscle and endothelial cells in inflamed mucosa. By contrast minimal MCP-1 mRNA expression and protein were found in histologically normal mucosa (Grimm *et al.* 1996).

RANTES, another monocyte attracting chemokine was found increased in the intestinal mucosa of patients with IBD compared with controls. The gene coding for RANTES was expressed in intraepithelial lymphocytes and in the lamina propria (Mazzucchelli *et al.* 1996a). Using in situ hybridisation, another study demonstrated that the monocyte attracting chemokines, RANTES, MIP-1α, MIP-1β, and γ-interferon-inducible protein-10 were expressed by macrophages, T lymphocytes, and endothelial cells in actively inflamed tissue but rarely expressed in uninflamed sections from IBD. The frequency of chemokine-expressing cells was significantly greater in severely inflamed than in moderately or mildly inflamed tissue. RANTES was expressed by T lymphocytes in normal colon lamina propria, although infrequently (Grimm & Doe, 1996). The increased number of MCP-1 and RANTES mRNA-expressing cells in mucosa from patients with IBD suggests that these cytokines play a role in the recruitment of peripheral blood monocytes and the expression of the MCP-1 gene in vessel-associated cells may indicate its involvement in

mechanisms regulating the adhesion of blood monocytes to endothelial cells (Mazzucchelli *et al.* 1996a).

Eotaxin is a directly chemoattractant cytokine for eosinophils, but not mononuclear cells or neutrophils. Eotaxin messenger RNA was found markedly accumulated in the lesions of patients with IBD, but not in the lesions of patients with diverticulitis. These results now provide a mechanism involving eotaxin to explain the eosinophil infiltration seen in IBD (Garciazepeda *et al.* 1996).

These data indicate an implication of colonic epithelial cells in chemokine production and from their post may be of paramount importance in the early recruitment of inflammatory and immune cells in intestinal inflammation.

1. 4. NITRIC OXIDE

In recent years, nitric oxide (NO), a gas previously considered a potentially toxic chemical, has become established as a diffusible universal messenger mediating cell-cell communication throughout the body (Moncada *et al.* 1991; Whittle, 1995). This molecule is a highly reactive free radical with a multitude of organ specific regulatory functions. NO is synthesised from the amino acid L-arginine by a family of enzymes (Figure 1) generally referred to as the nitric oxide synthases (NOSs). The oxidation of a terminal nitrogen of the amino acid L-arginine produces NO and L-citrulline. NO is a short-lived molecule and decomposes into other nitrogen oxides such as nitrite (NO2-) and nitrate (NO3-), and in the presence of superoxide anion to the potent oxidising agent peroxynitrite (ONOO-) (Fukuto & Chaudhuri, 1995). Three isoforms have been identified. Two of NOS are continuously present and are termed constitutive NOS (cNOS), while a third isoform is expressed after induction by certain cytokines, microbes, and bacterial products, thus it is called inducible nitric oxide synthase (iNOS) (Knowles & Moncada, 1994). All known

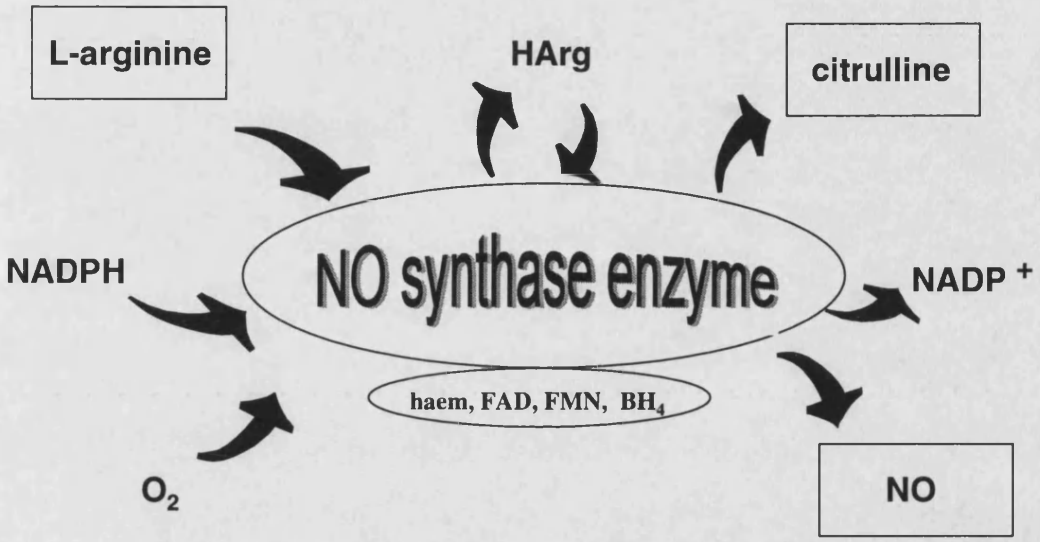

Figure 1. Nitric Oxide (NO) production from the aminoacid L arginine by the family of enzymes named Nitric Oxide Synthases (NOS)

isoforms of NOS require reduced nicotinamide adenine dinucleotide (NADH), flavine dinucleotide (FAD), flavine mononucleotide (FMN) and tetrahydrobiopterine (BH_4) as cofactors, but vary in their dependency for Ca^{2+} and calmodulin. Brain NOS, and endothelial NOS, which were cloned from rat cerebellum (Bredt *et al.* 1991), bovine and human vascular endothelial cells (Lamas *et al.* 1992; Janssens *et al.* 1992), respectively, are the constitutive enzymes (cNOS) and they are calcium and calmodulin dependent. Brain NOS and endothelial NOS share ~60% homology at the amino acid level. The third isoform, which is calcium and calmodulin independent was cloned from murine macrophages and it is ~50% homologous at the amino acid level to endothelial NOS (Lowenstein *et al.* 1992).

Constitutive NOS produce small amounts of NO and are involved in homeostatic processes (Moncada *et al.* 1991), while the inducible NOS is responsible for the main NO production in tissues and it is highly regulated by the action of cytokines (Moncada & Higgs, 1993). The soluble constitutive isoforms of NOS are activated by Ca^{2+}/calmodulin and no activity is observed in the absence of Ca^{2+}/calmodulin. NO production by cNOS is small, nM quantities, short lasting, it is controlled by Ca^{2+} mobilising agents in a very transient and highly controlled fashion, and fully inhibited by calmodulin antagonists (Stuehr & Griffith, 1992). In marked contrast iNOS synthesises NO in large (μM) amounts and is regulated at the transcriptional level (Morris & Billiar, 1994), although at the start of my study very little was known about human iNOS and the μM amounts of NO referred to rodent iNOS. NO production by iNOS is delayed by several hours following stimulation, but once induced, is active for hours and days. The gap between stimulation and enzyme generation might suggest the requirement of de novo synthesis of a co-factor, e.g. tetrahydrobiopterin (Stuehr & Griffith, 1992; Nussler *et al.* 1992) for maximal activity (Figure 1). iNOS is sensitive to inhibitors of DNA transcription and protein synthesis, such as actinomycin D and cycloheximide. Glucocorticoids (Radomski *et al.* 1990; Pfeilschifter, 1991) and cytokines such as TGFβ, PDGF, IL-8, IL-4 and IL-10 (Oswald *et al.* 1992; McCall *et al.*

1992; Ding *et al.* 1990; Cunha *et al.* 1992) have been found toinhibit iNOS activity, without affecting cNOS.

Based on early studies showing that N^{ω}-methyl-L-arginine blockedmacrophage mediated L-arginine-dependent tumour cell cytostasis (Hibbs *et al.* 1987b; Hibbs *et al.* 1987a), a variety of N^{ω}-substituted-L-arginine derivatives have been investigated as potential inhibitors of NOS. cNOS and iNOS also show some differences in their affinity for N^{ω}-monosubstituted arginine analogs, which compete with L-arginine for binding sites in the NO synthase enzymes and inhibit their activity (Gross *et al.* 199; Gross *et al.* 1990). Thus, N^{ω} -nitro-L-arginine (L-NNA) and N^{ω} -amino-L-arginine (L-NAA) are more potent inhibitors of cNOS and iNOS, respectively, while N^{ω} -mononethyl-L-arginine (L-NMMA) was found to be an effective inhibitor of both NOS types. The L-arginine analogues are selective inhibitors of NOS activity, in comparison to other known inhibitors (Knowles & Moncada, 1994). The human iNOS isoform has been cloned and its expression and activity has been induced in human hepatocytes (Geller *et al.* 1993), mesangial cells (Nicolson *et al.* 1993), lung epithelial cells (Robbins *et al.* 1994; Asano *et al.* 1994), monocytes/macrophages (Reiling *et al.* 1994), colonocytes Sherman *et al.* 1993), astrocytes (Lee *et al.* 1993), chondrocytes (Palmer *et al.* 1993; Charles *et al.* 1993) and smooth muscle cells (Junquero *et al.* 1992; Nakayama *et al.* 1994) stimulated with a "cocktail" of cytokines.

NO has numerous physiological and pathophysiological actions, having both anti-inflammatory and proinflammatory properties depending on the type and phase of the inflammatory reaction (Moilanen & Vapaatalo, 1995). In mammals, it is a recognised mediator of blood vessel relaxation that helps to maintain blood pressure (Welch *et al.* 1995) and vascular integrity (Cosentino & Luscher, 1995). In the central nervous system NO acts as a non-conventional neurotransmitter and participates in the establishment of long-term plasticity required for memory formation. This molecule is an important

regulator of cerebral vascular tone. Tonic production of NO maintains the cerebral vasculature in a dilated state. NO appears to be an important vasodilator during activation of neurons by excitatory amino acids, somatosensory stimulation and cortical spreading depression. Tonic production of NO appears to be critical in vasodilatation during hypercapnia, although NO may not directly mediate vasodilatation (Brian *et al.* 1996).

Induction of NO synthesis in many cell types as part of the host response to sepsis and inflammation. Induced NO can have a variety of effects which may be detrimental or beneficial during sepsis or inflammation, depending on amount, duration, and anatomic site of synthesis. As arginine is the only physiological nitrogen donor for NO synthesis, metabolism of this amino acid may play an important role in regulation of NO synthesis during sepsis (Morris & Billiar, 1994). NO is believed to play a role in various pulmonary physiological processes, such as bronchodilation (Jorens *et al.* 1993). In addition, NO is responsible for mediation of macrophage cytotoxicity (Jorens *et al.* 1995), inhibition of platelet aggregation, and for some parts of the host response to sepsis and inflammation and contributes to certain disease states (Moncada *et al.* 1991; Kuo & Schroeder, 1995). The mechanism for these diverse effects involves the local diffusion of NO from generator to target cells, which represents a novel singal transduction mechanism between cells.

Production of NO in large amounts by iNOS activity can inhibit key enzymes by nitrosylation of reactive groups, such as iron-sulphur centres (Fe-S) and thiol groups (-SH), which are essential for enzyme catalytic function (Molina Y Vedia *et al.* 1992; Cattell & Cook, 1993). The anti-proliferative effects of NO may be due to inhibition of DNA synthesis via inactivation of the ribonucleotide reductase enzyme. NO can also inhibit enzymes in the mitochondrial electron transport chain and citric acid cycle, which may account for the cytotoxic and cytostatic effects of macrophage-derived NO on tumour cells and micro-organisms. The cytotoxic activity of NO may play a role in tissue injury during inflammatory diseases, and the production of NO can lead to the generation of

more toxic radicals through its reaction with superoxide, forming peroxynitrite, which degrades to form hydroxyl radicals (Beckman *et al.* 1990). Peroxynitrite is considered a potent oxidant that reacts with proteins, lipids, and DNA, and it is a potent initiator of DNA strand breakage, which is an obligatory stimulus for the activation of the nuclear enzyme poly ADP ribosyl synthetase (PARS). The peroxynitrite-PARS pathway, by leading to cell necrosis or apoptosis, contributes to cellular injury in a number of pathophysiological conditions including shock and inflammation, pancreatic islet cell destruction, and diabetes, stroke, and neurodegenerative disorders, as well as the toxic effects of various environmental oxidants or cytotoxic drugs (Szabo, 1996). The body can protect against NO-mediated tissue damage by producing corticosteroids, growth factors and cytokines that are potent inhibitors of nitric oxide production (Laskin *et al.* 1994; Liew, 1995).

A number of strategies have emerged with regard to a pharmacological control of pathological NO production and new therapeutic approaches are studied, that may provide new means for clinical medicine (Pfeilschifter *et al.* 1996). Since the various isoforms of NOS are distributed in cells and tissues according to their function, there is the possibility that manipulation of NO levels can be accomplished by designing specific pharmacological agents targeted at a single NOS isoform (Fukuto & Chaudhuri, 1995).

1. 4. 1 The role of nitric oxide (NO) in inflammatory bowel disease

Nitric oxide (NO) is produced at many sites in the gastrointestinal tract and it is believed to take a part in physiological and pathological events (Whittle, 1994; Kubes & Wallace, 1995). Increasing evidence, which is based on studies in animal models as well as in man, indicates that NO may be involved in gastrointestinal inflammation and it has a pathogenetic role in IBD (Boughton-Smith, 1994). Recent studies in a number of inflammatory disease patients have shown a marked increase in NO synthase activity in

the inflamed mucosa from patients with active UC compared to uninflamed control tissue. The concentrations of citrulline, the co-product of NO synthase, were found higher in rectal biopsy specimens from patients with active UC than in those from patients with quiescent disease or normal histology. Citrulline concentrations were significantly lower in biopsy specimens incubated with L-NMMA than in those incubated with D-NMMA. These results suggest that the increased citrulline biosynthesis must be a consequence of NO synthase activity, which simultaneously produces NO (Middleton *et al.* 1993a). Studies of the activity of both cNOS and iNOS in colonic mucosa and muscle tissue from patients with active UC and CD compared to control uninflamed tissue revealed a substantial increase of Ca^{2+}-independent NO synthase activity in UC, characteristic of the inducible form of the enzyme. iNOS activity in colonic mucosa of UC patients was about 8-fold higher than the value in control mucosa, with no individual overlap. In CD patients mucosal NOS activity did not differ from control values. Finally there was no difference in NOS activity in colonic muscle between UC patients and controls (Boughton-Smith *et al.* 1993). Measuring serum nitrate levels they found that nitric oxide production was increased in both active UC and CD (Pool *et al.* 1995). Luminal gas sampled from the colons of patients with active UC and controls was analysed, using a chemiluminescence technique and NO concentrations were found more than 100 times higher in the patients than in the controls (Lundberg *et al.* 1994). Reynolds et al, 1995 (Reynolds *et al.* 1995a), using another molecule-specific technique had similar results with increased luminal NO concentrations in ulcerative colitis patients but not in controls. UC patients with blood visible in the rectum were negative for NO and they suspected that NO, which is avidly bound by haemoglobulin, was trapped in the lumen by the blood (Reynolds *et al.* 1995a). Luminal NO measurements may reflect NO production only in the superficial parts of the mucosa, since NO production in deeper mucosal layers is bound, e.g. by haemoglobulin in blood vessels, and therefore will not reach the lumen. These results suggest that the excess NO production in UC mainly occurs in very superficial mucosal layers (Lundberg *et al.* 1994).

The cellular source of NO and iNOS in models of IBD has received little attention to date (Alican & Kubes, 1996). Boughton-Smith et al., 1993 have found enhanced activity of the inducible form of nitric oxide synthase in the mucosa of patients with UC, but not CD and they suggested that this iNOS expression is responsible for the increased NO production in UC. However, the definition of iNOS, which based on the lack of Ca^{++} dependency, was not confirmed by either mRNA or protein data. Other studies using immunostaining and in situ hybridisation demonstrated high expression of iNOS localised to the surface epithelium and crypts in the mucosa from patients with UC (Reynolds *et al.* 1995b). Recently this enzyme was detected in colonic tissue including the epithelium from patients with active UC (Godkin *et al.* 1996), while Singer et al., 1996, using immunostaining, were found focal iNOS labelling localised in inflamed colonic mucosa in UC, CD, and diverticulitis, suggesting that iNOS expression is a feature of intestinal inflammation. There was also a similar, but not identical, distribution of nitrosylated protein.

Excess NO produced by the inducible enzyme may theoretically exacerbate the clinicopathological features of UC by direct cytotoxicity, activation of neutrophils (Ribbons *et al.* 1995), vasodilatation, reduced smooth muscle tone (Middleton *et al.* 1993b), increased production of nitrosamines (to cause cancer) (Ohshima & Bartsch, 1994), and interaction with superoxide to form the highly toxic peroxynitrite radical (Singer *et al.* 1996). In contrast, cNOS activity may have a protective role through vasodilatation, reduced platelet aggregation and inhibition of fluid secretion (MacNaughton, 1993). There is growing evidence that endogenous NO regulates mucosal barrier integrity under physiological conditions and counters the increase in mucosal permeability associated with acute pathophysiological states. The potential mechanisms of action for the protective effects of NO include maintenance of blood flow (Kubes & Wallace, 1995), inhibition of platelet and leukocyte adhesion and/or aggregation within the vasculature, modulation of mast cell reactivity, and scavenging of reactive oxygen

metabolites such as superoxide (Alican & Kubes, 1996). In experimental models, the protective effect of L-NAME indicates that NO contributes to tissue injury and that its modulation may be a novel approach to treat IBD (Rachmilewitz *et al.* 1995a). Methylprednisolone was found to decrease NO generation by cultured colonic mucosa and it has been suggested that NO synthase activity is induced during the culture and this steroid effect may relate to its therapeutic effect. (Rachmilewitz *et al.* 1995c). Inhibition of NO synthesis by an L-arginine analogue significantly ameliorated the extent of tissue injury in two models of experimental (Rachmilewitz *et al.* 1995b). The further study of the cellular source of NO in colonic mucosa and the development of selective inhibitors of NO synthase isoforms will provide more information on the complex role of NO in physiology and pathophysiology of intestinal mucosa and should reveal novel targets for therapeutic intervention of intestinal inflammation.

1.5 AIM OF THE STUDY

Our study is based on the hypothesis that the intestinal epithelial cells constitute an interface between the host and the environment and they have a crucial role as an outpost of the immune system located in the underlying gut mucosa. In this study we explore their involvement in immune and inflammatory reactions via the release of inflammatory mediators.

The objectives of the investigation were:

1. To explore the potential of human colonic epithelial cells to express inflammatory mediators, such as chemokines and nitric oxide.

2. To investigate the mechanisms involved in nitric oxide production and regulation in human colonic epithelial cells.

3. To characterise the spectrum of chemokine production by human colonic epithelial cells and to determine which factors can modulate this generation.

2. To explore the effects of T cell-derived cytokines on nitric oxide and chemokine production in human colonic epithelial cells.

The elucidation of the role of the colonic epithelial cells in inflammatory process could offer a novel approach in the development of new diagnostic tests and potential new therapeutic strategies for the effective treatment of intestinal inflammation.

2. MATERIALS AND METHODS

2.1 MATERIALS

Human recombinant IL-1α (specific activity $5x10^7$ U/mg), a generous gifts from Glaxo (Greenford, UK) was diluted in sterile PBS + 0.25% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C.

Human recombinant TNF-α (specific activity $6x10^7$ U/mg), a generous gifts from Bayer (Slough, UK) was diluted in sterile PBS + 0.1% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C.

Human recombinant IFN-γ (specific activity > $2.0x10^7$ U/mg) was purchased from Boehringer Mannheim, U.K and stored in aliquots at -70^0 C.

Human recombinant IL-10 (specific activity $1x10^7$ U/mg), kindly donated by Dr K.W. Moore (DNAX Palo Alto, California U.S.A.) was diluted in sterile PBS + 0.1% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C.

Human recombinant IL-4 (specificity > $1x10^7$ U/mg) was purchased from Genzyme and stored in aliquots at -70^0 C.

Human recombinant IL-13 was purified from culture supernatants of stable transfected CHO cells (Minty *et al.* 1993) and generously provided by Dr A. Minty (Sanofi, Recherche, Labege, France). IL-13 was diluted in sterile PBS + 0.25% (w/v) bovine serum albumin (BSA, low endotoxin) (Sigma) and stored in aliquots at -70^0 C.

All cell culture plastics were purchased from Nunc.

2,3-Diaminonaphthalene (DAN) was purchased from Lancaster Synthesis Ltd.

Cycloheximide, Sodium nitrite, Actinomycin-D, and Dimethyl-sulphoxide (DMSO) were purchased from Sigma Chemical, UK.

The Digoxigenin (DIG) chemiluminescent detection kit for Northern blotting was from Boehringer Mannheim (Lewes, U.K.).

5'Digoxigenin labelled probe for iNOS was cocktail containing 3 antisense 30-mer oligonucleotides purchased from R & D Systems (Abingdon, U.K.).

5'Digoxigenin labelled probes for IL-8, MCP-1, and RANTES were cocktails containing 3 and 4 antisense 30-mer oligonucleotides, respectively, purchased from R & D Systems (Abingdon, U.K.).

5'Digoxigenin labelled probes for β-actin was cocktail containing 4 antisense 30-mer oligonucleotides, purchased from R & D Systems (Abingdon, U.K.).

Antibodies for IL-8 (anti-human IL-8 mouse monoclonal antibody, alkaline phosphatase conjugated anti-human IL-8 goat polyclonal antibody and human recombinant IL-8 standard) enzyme-linked immunosorbent assay (ELISA) were a generous gift from Dr I.J.D. Lindley (Sandoz Forschungsinstitut, Vienna, Austria) (Ceska *et al.*, 1989).

Antibodies for RANTES (anti-human RANTES mouse monoclonal antibody and anti-human RANTES goat polyclonal antibody) were purchased from R & D Systems (Abingdon, UK). In ELISA, this antibody does not cross-react with other chemokines tested (R & D Systems).

Antibodies for MCP-1 (anti-human MCP-1 mouse monoclonal antibody and polyclonal rabbit aMCP-1), were a gift from Dr. T. Yoshimura (NIH) (Yoshimura *et al.*, 1991).

Dr J.R. Weinder and colleagues at Merck Research Laboratories, Rahway, New Jersey, U.S.A, characterised and generously provided a **rabbit anti-iNOS affinity purified IgG designated NO-53**, which was raised against the last seven carboxy terminal amino acids of human iNOS and does not cross react with the constitutive NOS isoform (Singer *et al.* 1996) and the **immunogenic peptide** (H_2N-Tyr-Arg-Ala-Ser-Leu-Glu-Met-Ser-Ala-Leu-Ser-COOH), as specificity control.

Mouse monoclonal anti-human IL-8 for immunostaining was a generous gift from Dr I.J.D. Lindley (Sandoz Forschungsinstitut, Vienna, Austria).

The **ABC complex** and the **DAB (3,3'-Diaminobenzidine)** peroxidase substrate tablets set were from DACO and Sigma, respectively.

2.2 CELL CULTURE CONDITIONS

2.2.1 HT-29 colon adenocarcinoma cell line

The human colon epithelial cell line HT-29 was obtained from the European Collection of Animal Cell Cultures (ECACC). HT-29 cells are human colon adenocarcinoma grade II cells isolated from a primary tumour in a 44 year old Caucasian female (ECACC). They are a well characterised epithelial cell line and have characteristics of normal intestinal epithelium such as epithelial polarity, presence of the actin-biding protein villin and the occurrence of an enterocytic differentiation (Chantret *et al.* 1988).

2.2.2 Cell culture

HT-29 cells were routinely cultured in 80 cm^2 tissue culture flasks in McCoy's medium supplemented with penicillin (10u/ml), streptomycin (10µg/ml), fungizone (0.5µg/ml), and 10 % (v/v) FCS (referred to as complete medium). Cultures were maintained at 37^0 C

in an atmosphere of 5 % CO_2. The medium was changed every 3 days. To subculture confluent monolayers, the medium was removed and the cells were washed 3 x with PBS (w/o Ca^{2+} and Mg^{2+}). Cells were then washed (1X) with a 3ml Trypsin-EDTA mixture of 0.05% (w/v) Trypsin and 0.02% (w/v) EDTA. The excess solution was removed and the cells were incubated for approximately 5 mins at 37^0 C until the cells had detached from the flask. The action of trypsin/EDTA was inhibited by adding 10ml of complete McCoy's medium and the cell suspension was centrifuged at 200 g for 5 min. The cell pellet was resuspended in complete medium and cell counting and viability were checked in a Neubauer haematocytometer after mixing with Trypan Blue (Sigma). Dead cells stained blue, due to the uptake of Trypan Blue. Cell viability was always greater than 95%. Cells were counted and then seeded at 2-3 X 10^4 / ml of McCoy's complete medium, into 80 cm^2 tissue culture flasks for further culture, or into 6-well plates for experimental protocols. Flasks and plates reached confluency after approximately 6 days. For storage, cells were resuspended at 4 X 10^6 cells /ml of freeze medium. The freeze medium contained 10% of dimethylsulphoxide (DMSO) (Sigma), 40% FCS, and 50% McCoy's medium. The cell suspension was transferred to cryotubes (Nunc) at 1 ml / tube, gradually cooled in vapour phase of liquid nitrogen overnight and tubes were stored in liquid nitrogen tanks. For resuscitation of cells from liquid nitrogen, cells were rapidly defrosted at 37^0 C in a water bath, washed in McCoy's medium, resuspened in complete medium and cells from 1 cryotube were seeded into 80 cm^2 tissue culture flasks in McCoy's medium, continuing as above.

2.2.3 Mycoplasma assay

HT-29 cells were tested for contamination with the 4 common mycoplasma species (M. arginini, M. hyorhinis, A. laidlawii, M.orale) using an enzyme immunoassay kit

(Boehringer Mannheim). The protocol recommended by the manufacturers was followed and 4 day old culture supernatants were routinely screened. The protocol was used as follows. 96 well microtitre plates (Nunc Immuno maxisorb plates) were coated with 125 µl of four different antibody solutions directed against the four mycoplasma species. Plates were incubated for 2 hours at 37^0 C. Antibody solutions were removed and non specific binding sites blocked with 125 µl of blocking solution for 30 minutes at 37^0 C. Plates were washed 3 x and incubated with 100 µl of either cell culture supernatant, or positive control solutions, or medium alone, at 4^0 C overnight. Plates were washed 3 x and incubated with 100 µl of the four corresponding detecting biotin-labelled antibodies for 2 hours at 37^0 C. Plates were washed 3 x and incubated with 100 µl of streptavidin-alkaline phosphatase for 1 hour at 37^0 C. Washed plates were incubated with 100 µl of substrate solution for 1 hour at room temperature. Results were evaluated visually and confirmed by reading optical densities at 405 nm. All cultures tested during the study were negative for all four mycoplasma species were examined.

2.3 EXPERIMENTAL PROTOCOL

HT- 29 cells were grown in 6-well plates until confluent. Twenty-four hours prior to the experiment the confluent monolayers were washed and cultured in FCS-free McCoy's medium. Growth arrested cultures were then treated with fresh FCS-free medium and stimulated with the appropriate doses of either drugs, or cytokines, or vehicle controls for the times described in the results section. Supernatants were collected, centrifuged to remove cellular debris and stored at -70^0 C until assayed for extracellular chemokines or nitrite. Total RNA and cellular proteins were extracted as described below. Cell counting and viability was routinely checked at the beginning and the end of the experiment, by

phase microscopy and by trypan blue exclusion, using representative wells. Cell viability was always greater than 95%.

2.4 COLONIC BIOPSY CULTURES

2.4.1 Patients

Colonic biopsies were selected from patients who had undergone colonoscopy at the Royal United Hospital, Bath. The patient group (n=8, 3 men and 5 women, median age 55.6 years, range 42-74) included patients with diverticular disease (n=3), colon adenocarcinoma (n=2), and normal individuals (n=3). These samples in routine histology had normal morphology and number of glands, normal numbers of goblet cells and did not show inflammatory infiltration of the lamina propria. Patients consent was obtained and the local Research Ethics Committee granted approval for this study.

2.4.2 Biopsy culture

Human colonic biopsies, after removal from patient, were immediately placed in transport medium, Hanks' balanced salts solution (HBSS) pH 7.4, supplemented with antibiotics (penicillin 100U/ml, streptomycin 100 μg/ml, gentamycin 50 μg/ml, and fungizone 2.5 μg/ml), transferred to the laboratory, and gently washed 3 X of 15 minutes in the transport medium. Mucosal biopsy specimens were then placed in a six-well plate (Gibco), containing 2 ml of Trowell's-T8 65% (v/v) / RPMI-1640 25% (v/v) / foetal calf serum 10% (v/v) medium (supplemented with Hepes buffer 10 mM, glutamine 2 mM, penicillin 100U/ml, streptomycin 100 μg/ml, gentamycin 50 μg/ml, and fungizone 2.5 μg/ml) per well. Colonic biopsies were incubated at 37^0 C in an atmosphere of 5% CO_2 in the presence of appropriate stimuli and were cultured for 30 hours. Supernatants were

then collected for nitrite measurement and colonic specimens were used for total protein estimation of each well.

2.4.3 Protein assay

After 30 hours culture with appropriate stimuli culture supernatants removed for nitrite measurement and colonic biopsy specimens were used for estimation of total protein per well, using the Bio-Rad Protein microassay. The protein assay was based on the Bradford dye-binding procedure (Bradford, 1976). Known concentrations of bovine serum albumin (BSA) (Sigma) diluted in phosphate buffered saline (PBS), pH 7.4 were used as a standard curve. Colonic specimens were homogenised in PBS, pH 7.4, using an ultrasonic homogeniser and solubilised tissue was diluted in various dilutions (1:10-1:200). 200 µl of sample or standard and 50 µl of Bio-Rad protein Assay (Bio-Rad) were added per well in a 96-well microtiter plate and protein was measured with a microplate reader. Nitrite production was expressed as pmol/mg of protein.

2.5 ENZYME-LINKED IMMUNOSORBENT ASSAYS (ELISA)

Extracellular chemokine activity of culture supernatants was measured by double ligand ELISA (Brown *et al.* 1994). All ELISA samples were measured in duplicate

2.5.1 IL-8 ELISA

96 well microtitre plates (Nunc immuno maxisorb plates) were coated with 100 µl/well of monoclonal anti-IL-8 antibody (5 µg/ml) in carbonate coating buffer. Plates were covered and incubated overnight at 4^0 C. Plates were washed 3 x with wash buffer, followed by addition in duplicate of 100 µl IL-8 standard (0.05 to 2 ng/ml) or culture supernatant

appropriately diluted in wash buffer + 2 % (v/v) FCS, and incubated for 2 hours at 37^0 C. Plates were washed 3 x and 50 µl alkaline phosphatase-conjugated goat anti-IL-8 antibody (5 µg/ml) in wash buffer + 2 % FCS was added for 2 hours at 37^0 C. Following 3 washes, plates were incubated with 100 µl of 1 mg/ml *p*-nitrophenyl phosphate in warmed diethanolamine buffer at room temperature, until the top standard read ~ 1.5 OD. The reaction was terminated with 50 µl / well 3M NaOH, and the OD measured at 405nm. The assay was linear between 0.2-2 ng/ml.

2.5.2 RANTES ELISA

96 well microtitre plates (Nunc immuno maxisorb plates) were coated with 50 µl/well of anti-RANTES monoclonal antibody (1 µg/ml) in carbonate coating buffer, pH 9.6. The plates were covered and incubated overnight at 4^0 C. Plates washed 3 x with wash buffer, followed by the addition in duplicate of 50 µl of RANTES standard (0.1 to 6 ng/ml) or culture supernatant diluted in wash buffer + 2 % FCS, and incubated for 2 hours at 37^0 C. Plates were washed 3 x and 50 µl biotynolated anti-RANTES polyclonal antibody (1 µg/ml) in wash buffer + 2 % FCS added for 2 hours at 37^0 C. Plates were washed 3 x and incubated with 50 µl streptavidin-peroxidase (0.5 mg/ml) in wash buffer + 2 % FCS for 30 minutes. Following 3 washes, 100 µl of 0.2 mg/ml OPD dissolved in warm 0.1 M citric acid-phosphate buffer containing 0.4 µl/ml 30 % (v/v) H_2O_2 was added. After a 20 minutes incubation in the dark, at room temperature, the reaction was quenched with 150 µl 1 HSO and optical densities (OD) at 492 nm were determined. The assay was linear between 0.2-4 ng/ml.

2.5.3 MCP-1 ELISA

96 well microtitre plates (Nunc immuno maxisorb plates) were coated with 100 µl/well of monoclonal anti-MCP-1 antibody (4.5 µg/ml) in coating buffer. Plates were covered and incubated overnight at 4^0 C. Vacant sites on the plastic were blocked with 0.3 ml/well of 0.2% BSA in coating buffer and plates were incubated at least for 30 min at 37^0 C. Plates were then washed 3X with wash buffer, followed by addition in duplicate of 100 µl of serially diluted MCP-1 standard (0.05 to 10 ng/ml) or culture supernatant appropriately diluted in TBS-Tween-0.2% BSA, and incubated for 90min at 37^0 C. Plates were washed 3X with wash buffer and 100 µl/well of polyclonal rabbit anti-MCP-1, diluted in TBS-Tween-0.2% BSA was added for 90 min at 37^0 C. Following 3 washes, plates were incubated with 100 µl/well of alkaline phosphate labelled anti-rabbit IgG in TBS-Tween for 90 min at 37^0 C. Plates were washed 3X with TBS w/o Tween and followed by addition of substrate solution (5mg Sigma 104 phosphate in 5ml substrate buffer) and after 20 min at room temperature the reaction was stopped with 0.1ml/well of 3N NaOH. The OD measured at 405nm. The assay was sensitive down to 0.2ng/ml.

2.6 NORTHERN ANALYSIS

Total cellular RNA was isolated from HT-29 colon adenocarcinoma cells using a modification of the method of Strieter *et al.,* 1989. Buffers used for northern analysis are detailed below, and all solvents were "analytical reagent" grade.

2.6.1 RNA isolation

HT-29 cells, in six well plates, were scraped into 1.5 ml nucleic acid extraction buffer and frozen at -70^0 C in 15 ml sterile polypropylene conical tubes (Falcon) for at least 1 hour,

preferably overnight. For extraction the cell mixture was defrosted and homogenised by pipeting up and down 25 x using a sterile pastette. 1.5 ml of phenol extraction buffer and 1.5 ml of an 1:1 solution of water-saturated phenol (Rathburn Chemicals Ltd, Walkerburn, UK) : chloroform was added and each tube vortexed vigorously for 2 minutes. Tubes were centrifuged at 3,000 g for 10 minutes at 4^0 C. Using a sterile glass pipette, the upper aqueous layer was transferred to a fresh 15 ml tube and 1.5 ml phenol : chloroform (1:1) added. The tubes were vortexed and centrifuged as before. The upper aqueous layer was transferred to a fresh 15 ml tube and 2.5 ml of 1:25 solution of isoamyl alcohol : chloroform added. The tubes were vortexed and centrifuged at 3,000 g for 7 minutes. The upper aqueous layer was transferred to a fresh 15 ml tube and mixed with 300 µl 3M sodium acetate and 3 ml of isopropyl alcohol (Fisons propan-2-ol, HPLC grade). Tubes were kept at -70^0 C for at least 1 hour to allow RNA to precipitate. The contents were then transferred to a 16 x 16 mm polyallomar ultra centrifuge tube (Beckman Instruments Ltd, Buckinghamshire, UK), which had been DEPC-treated and autoclaved. The tubes were centrifuged at 30,000 g for 75 minutes at 4^0 C. The supernatants was removed and RNA pellet transferred to an Eppendorf tube in 1.5 ml 75 % ethanol solution. The Eppendorfs were frozen at -70^0 C for at least 1 hour, or until ready to run gels.

2.6.2. Sample preparation

Eppendorfs containing the RNA samples were defrosted and centrifuged at 12,000 g for 15 minutes at 4^0 C. The supernatant was removed and 1.5 ml 75 % ethanol added to the RNA pellet. The samples were centrifuged and the wash step repeated twice. The supernatant was removed and the pellets air dried in a fume cupboard for 1 hour. RNA pellets were resuspended in 50 µl DEPC-treated water and kept on ice. RNA was

quantitated by measuring absorbance of 2 µl RNA in 1 ml 0.1 M NaOH at 260 nm. The amount of RNA present in the samples (in µg) was calculated by:

A_{260} x dilution factor (500) x 40 x volume of remaining RNA solution in ml (0.048)

ODs were also read at 280 nm and 230 nm to assess the purity of RNA. A value of less than 2 for the OD_{260} : OD_{280} ratio indicated protein contamination. A low OD_{260} : OD_{230} ratio indicated guanidine contamination. 30 µl of RNA sample buffer was added to 10 µg RNA and the samples were vortex mixed and heated for 15-30 minutes at 80^0 C. The samples were cooled on ice and 2.5 µl of bromophenol blue solution were added. Samples were mixed and briefly centrifuged (5 secs) prior to loading on agarose gels.

2.6.3. Gel preparation and transblotting

1 % agarose gel was prepared by dissolving 3 g agarose (Boehringer Mannheim) in 230 ml DEPC-treated water and the solution was heated in microwave oven until the agarose dissolved. 15 ml 20 x MOPS buffer and 54 ml formaldehyde were added and the gel solution allowed to cool to ~ 60^0 C before pouring. The gel was set with two 15 lane combs using tanks purchased from Hoefer Scientific Instruments (Newcastle, UK). After 40 minutes, the gel was transferred to a submarine tank (Hoefer), which was surrounded with ice, and covered with cold 1X MOPS running buffer. 10 µg RNA per lane was loaded and the gel run at constant current of 100 mA, until the bromophenol blue band had migrated 1.5 inches (~ 2 hours). The gel was placed under a UV light and the ethidium-bromide stained 18S and 28S ribosomal RNA bands observed to assess equal loading. The gel was photographed using a polaroid CU5 88-46 land camera (Genetic research instrumentation Ltd) and type 55 polaroid film (Sigma). The gel was agitated gently in DEPC-treated water for 30-60 minutes to remove formaldehyde, prior to transblotting. The blotting tank consisted of a glass plate suspended in a sandwich box,

which was half filled with 20 x SSC buffer. A wide strip of filter paper placed over the glass plate and reaching down into the buffer solution at each end acted as a wick. The gel was placed upside down on the filter paper and covered with a piece of positively charged nylon membrane (Boehringer Mannheim), which had been briefly soaked in 20 x SSC. All air bubbles were removed by rolling with a sterile glass pipette, before covering the membrane with 3 pieces of similar size paper and a stack of paper towels. A 500 g weight was placed on top and left overnight to allow the RNA to transfer by capillary action. The RNA was fixed onto the nylon membrane by baking in an vacuum oven (Jouen) at 120^0 C for 20 minutes. The membrane was sealed in a plastic bag and stored at room temperature prior to hybridisation.

2.6.4. Hybridisation with DIG-labelled oligonucleotides

Hybridisation of membranes and detection of bound probes was performed essentially as described in the Digoxigenin (DIG) chemiluminescent detection kit for Northern blotting of Boehringer Manheim. The DIG detection system is based on the labelling of nucleid acid probes with a steroid hapten, digoxigenin. The DIG-labelled probes are hybridised to membrane-bound RNA. Specific hybridisation is immuno-detected with an alkaline phosphatase conjugated anti-digoxigenin antibody and visualised with the chemiluminescent substrate, CSPD, using X-ray film. Hybridisation temperature was optimised for each probe. IL-8, MCP-1, RANTES, and β-actin probes were all hybridised at 42^0 C, while the iNOS probe was used at 60^0 C. All probes were used at a final concentration of 10 ng/ml. The volumes specified are for a 100 cm^2 membrane and pre-hybridisation, hybridisation and wash steps were all performed at the appropriate hybridisation temperature. The membrane was prehybridized by incubating with 20 ml hybridisation solution for 1 hour in a sealed bag. The hybridisation solution was

discarded and 2.5 ml of probe diluted to 10 ng/ml in hybridisation solution was added. All air bubbles were removed and the bag resealed and incubated overnight. The membrane was transferred to a small sandwich box and washed for 2X of 5 min in 2 x SSC, 0.1 % SDS solution followed by 2X of 5 minutes in 0.1 x SSC, 0.1 % SDS solution at hybridisation temperature. The following steps were then performed at room temperature on a shaking water bath. Membranes were washed for 5 minutes in wash buffer, prior to blocking for 30 minutes with 100 ml buffer 2. Membranes were incubated for 30 minutes with 20 ml alkaline phosphatase conjugated anti-DIG antibody diluted 1 : 10,000 in buffer 2. Membranes were washed 2X of 15 min in wash buffer and equilibrated for 2-5 min in buffer 3. The membrane was drained and incubated for 5 min between 2 plastic sheets with 1 ml of lumigen PPD substrate diluted in 1 : 100 in buffer 3. The membrane was drained again, sealed in a plastic bag and incubated for 15 min at 37^0 C in a dark place. The membrane was then exposed to Kodak omat AR5 X-ray film (Sigma) for appropriate time at room temperature.

2.7 FLUOROMETRIC NITRITE ASSAY

Nitric oxide (NO) was determined by measuring in culture supernatants the stable-end product nitrite. Nitrite was measured using a fluorometric assay based upon the reaction of 2,3- diaminonaphthalene (DAN) (Lancaster, Morecambe, UK) with nitrite under acidic conditions to form the fluorescent product 1-(H)- naphthotriazole. The assay was modified for use on a Photon Technology International (PTI) spectrofluorimeter from the method of Misko *et al* (Misko *et al.* 1993), which employed a 96 well plate format for a fluorescent plate reader. In contrast to the plate reader, the optimum excitation and

emission wavelengths for the assay could be set on the fluorimeter, thus improving sensitivity. Fluorescent excitation and emission spectra for 1-(H)- naphthotriazole were obtained as previously described (Misko *et al.* 1993) and optimum wavelengths were determined. An excitation wavelength of 365 nm and emission wavelength of 405 nm were found to be optimum, which was consistent with previous findings (Misko *et al.* 1993). A standard curve of sodium nitrite in McCoy's medium ranging from 100 nM to 2 µM was prepared. 2 ml of standard or culture supernatant was mixed in a bijoux with 200 µl of freshly prepared DAN reagent (0.05 mg/ml DAN in 0.62 M HCl) and incubated at room temperature in the dark. After 10 minutes the reaction was stopped by the addition of 100 µl 2.8 N NaOH. Fluorescent intensity of 2 ml volumes of standards and samples was measured on the fluorimeter using an excitation wavelength of 365 nm and an emission wavelength of 405 nm. Phenol red present in McCoy's medium did not interfere with the assay. The sensitivity of the assay was 10nM.

2.8 WESTERN BLOT ANALYSIS

2.8.1 Collecting samples

HT-29 cells, after incubation with appropriate stimuli, were extracted for iNOS protein by scraping the cell monolayer into 0.3 ml of 1X sample buffer. 1 µl of 5 µg/ml carboxypeptidase inhibitor (Sigma) was added per ml of sample buffer. Samples were immediately boiled for 5 minutes (100^0 C), and then stored in 100 µl aliquots at -20^0 C, until required. Before use samples were centrifuged at 12,000 x g for 2 min.

2.8.2 Separation of cell proteins for iNOS detection

Proteins were analysed by one dimensional gel electrophoresis, which under reducing conditions separates proteins based on molecule size. Sodium Dodecyl Sulphate - Polyacrylamide Gel Electrophoresis (SDS-PAGE) was carried out essentially as described by Laemmli (Laemmli, 1970). Proteins were separated by SDS-PAGE using the Protean II xi gel system (Bio-Rad).

Minigels (5 % (w/v) acrylamide stacking gel and 7 % (w/v) acrylamide running gel) were prepared as described in buffers and solutions section.

The running gel was poured into the gel equipment and overlaid with a layer of 50% methanol in distilled water. After 1 hour the methanol was removed the top of the gel was rinsed with distilled water and the stacking gel was poured on the top of the running gel and set with a 15 lane comb. Samples were thawed and centrifuged at 12,000 g for 2 minutes prior to loading. When stacking gel was set the comb was removed and 20 µl of sample was loaded per lane (~ 20 µg) of stacking gel and a prestained molecular weight marker was included on each gel. Gels were run at room temperature at 100 volts, until the bromophenol blue tracking dye entered the running gel. The voltage was then increased to 150 volts. Gels were run until the bromophenol blue band had reached the bottom of the resolving gel. Gels were then placed into transfer buffer in preparation for Western blotting.

2.8.3 Electrophoretic transfer of proteins to nitro-cellulose

Solubilized roteins after separation on 7 % polyacrylamide gels were transferred to nitro-cellulose paper, using a transblot electrophoretic apparatus (Bio-Rad). The polyacrylamide gel was soaked for 15 minutes in transfer buffer and the lanes of the stacking gel removed. Two pieces of filter paper, a piece of nitro-cellulose paper, just

slightly larger than the gel and two nylon pads were also soaked in transfer buffer. A sandwich was constructed in which the gel was overlaid with a piece of wet nitro-cellulose membrane (Protein BA 85, Schleicher and Schuell, Dassel, Germany), taking care to remove all air bubbles and both were surrounded on each side by a layer of filter paper and a nylon pad. The sandwich was enclosed in a cassette and placed in the transfer tank (Bio-Rad) with the nitro-cellulose nearest the anode. The tank was filled with cold (4^0 C) transfer buffer and placed on a magnetic stirrer. Electrophoretic transfer was carried out for 35 minutes at 75 volts.

2.8.4 Immunoblotting of nitro-cellulose-bound protein

Western blot analysis of iNOS protein was performed according to the method of Dr J. Weinder, MERCK research laboratories, Rahway, NJ (personal communication). NO53 rabbit antisera directed against the C-terminal sequence of human iNOS and control NO54 blocking peptide were gifts from MERCK. Antisera were diluted 1 : 1 in glycerol and stored at -20^0 C for daily use. Non specific binding sites on the nitrocellulose were blocked for 30 min with 3% non fat-dried milk protein reconstituted in deionized water. After washing the nitrocellulose membrane for 10 min in PBS-T (PBS w/o Ca^{++} and Mg^{++} containing 0.05% Tween-20) the membranes were incubated for 1 hour with rabbit anti-human iNOS antibody (NO-53) diluted 1:40,000 in PBS-T. The membranes were then washed (3 x 10 min) in PBS-T before incubation for 1 hour with sheep anti-rabbit IgG conjugated to alkaline phosphatase. After further washing in PBS (3 x 10 min) iNOS protein was detected using 5-bromo-4-chloro-3-indolyl phosphate substrate solution (Sigma). The reaction was terminated with 4 mM EDTA in PBS. To avoid carryover, all incubations except the wash steps were performed in separate containers. To establish the specificity of anti-iNOS antibody (NO-53) additional Western blots were probed with NO-

53 IgG in the presence of the immunogenic peptide (H_2N-Tyr-Arg-Ala-Ser-Leu-Glu-Met-Ser-Ala-Leu-Ser-COOH) at concentrations 50 nM. All incubations and washes were carried out at room temperature with gentle shaking.

2.9 IMMUNOHISTOCHEMICAL STUDY OF COLONIC MUCOSA

2.9.1 Patients

Colonic biopsies were selected from patients who had been diagnosed at the Royal United Hospital, Bath. The patient groups included active ulcerative colitis (n=12, 9 men and 3 women, median age 42 years, range 24-59), infectious colitis (n=3, 3 men, median age 41 years, range 29-56), and controls (n=10, 6 men and 4 women, median age 64 years, range 26-84). All ulcerative colitis patients were newly diagnosed with moderate to severe disease activity at the time of colonoscopy. All patients were examined before treatment and biopsy samples were taken from the areas of greatest involvement of the inflammatory process. Routine histology of these specimens demonstrated ulceration, mucin depletion, deformation of the glands, crypt abscesses, and inflammatory cell infiltration of the lamina propria. Patients with infectious colitis, due to salmonellosis were examined and biopsies were taken before treatment and in total remission after treatment. Biopsies of normal bowel mucosa from patients with diverticular disease (n=2), colon adenocarcinoma (n=6), and normal individuals (n=2) were used as controls. These samples in routine histology had normal morphology and number of glands, normal numbers of goblet cells and did not show inflammatory infiltration of the lamina propria. Patients consent was obtained and the local Research Ethics Committee granted approval for this study.

2.9.2 Immunohistochemistry for iNOS

Polyclonal rabbit antibody against the C-terminal sequence of human iNOS was used in an avidin-biotin method to demonstrate the presence of iNOS. Colonic biopsies which had been fixed in formalin and embedded in paraffin were mounted on APES coated slides. The mounted slides were deparaffinized with xylene. Endogenous peroxidase was blocked in 2.5% hydrogen peroxide in methanol for 10 min and rehydrated in distilled water for 5 min. The slides were then washed with Tris buffered saline, pH 7.6 (TBS), treated with 5 % normal swine serum for 5 min, and exposed to rabbit anti-human iNOS (NO-53), diluted 1:1000. After an 1 hour incubation at room temperature the sections were rinsed with TBS, incubated for 40 min in swine anti-rabbit biotynolated secondary antibody, diluted 1:500, and washed in two changes of TBS. Slides were then treated with avidin-biotin complex for 40 min, rinsed twice in TBS, overlaid with 3,3'-diaminobenzidine (DAB) peroxidase substrate and incubated for 10 min at room temperature to allow for colour development. Gills haematoxylin was used as a counterstain. Blocking experiments were carried out by adding 150 nM of the immunogenic peptide (H_2N-Tyr-Arg-Ala-Ser-Leu-Glu-Met-Ser-Ala-Leu-Ser-COOH) to the working dilution of anti-iNOS antibody.

2.9.3 Immunostaining for IL-8

Mouse monoclonal antibody against the human IL-8 was used in an avidin-biotin method to demonstrate the presence of IL-8. Colonic biopsies which had been fixed in formalin and embedded in paraffin were mounted on APES coated slides. The mounted slides were deparaffinized with xylene. Endogenous peroxidase was blocked in 2.5% hydrogen peroxide in methanol for 10 min and rehydrated in distilled water for 5 min. The slides were then washed with TBS, treated with 5 % normal rabbit serum for 5 min, and exposed to mouse anti-human IL-8, diluted 1:100. After an 1 hour incubation at room temperature the

sections were rinsed with TBS, incubated for 40 min in rabbit anti-mouse biotynolated secondary antibody, diluted 1:400, and washed in two changes of TBS. Slides were then treated with avidin-biotin complex for 40 min, rinsed twice in TBS, overlaid with DAB peroxidase substrate and incubated for 10 min at room temperature to allow for colour development. Gills haematoxylin was used as a counterstain.

2.10 STATISTICAL ANALYSIS

Triplicate determinations were performed in each experiment. The (n) number of each experiment is given in the respective figure legend. Data were analysed by two-way analysis of variance (ANOVA) to determine if any statistical significance existed within the data groups. ANOVA was followed by Dunnett's test for the comparison of multiple groups to controls. This latter test identified which treatments within the group were significantly different from the control. Data were expressed as means ± SEM of (n) experiments. A probability value of $p < 0.05$ was taken as the criterion for a significant difference.

2.11 BUFFERS AND SOLUTIONS

2.11.1 Solutions and reagents for cell and tissue culture

Tissue culture reagents were used for cell and tissue cultures and all solution were prepared using sterile, pyrogen-free distilled water (Steripak Ltd, Cheshire,UK).

Phosphate Buffered Saline (PBS), pH 7.4

140 mM NaCl

2.7 mM KCl

1.5 mM KH_2HPO_4

8.1 mM Na_2HPO_4

Hanks' Balanced Salts Solutions (HBSS), pH 7.4

100 ml 10X Hanks' balanced salts (Gibco)

6 ml 7.5 % (w/v) sodium bicarbonate (Gibco)

20 ml 1M HEPES buffer solution (Gibco)

200 µl 40% (w/v) NaOH

Make up to 1L with distilled water. No need to pH.

McCoy's medium 5A (Gibco)

Supplemented with penicillin (10u/ml, Gibco), streptomycin (10 µg/ml) (Gibco), and fungizone (0.5 µg/ml) (Gibco) before use.

Trowell's T8 / RPMI-1640 medium

65% Trowell's T8 (Gibco)

25% RPMI-1640 (Gibco)

10% Foetal Calf Serum (FCS) (Gibco)

10 mM HEPES buffer solution (Gibco)

2 mM Glutamine (Gibco)

Supplemented with penicillin (10U/ml) (Gibco), streptomycin (10 µg/ml) (Gibco), and fungizone (0.5 µg/ml) (Gibco) before use.

Foetal Calf Serum (FCS)

FCS (Gibco) was heat-inactivated at 56^0 C for 30 minutes and stored at -20^0 C.

2.11.2 Solutions and buffers for Nitrite assay

HCl solution

0.62 M HCl

in Milli-Q water.

NaOH solution

2.8 M NaOH

in Milli-Q water.

2.11.3 Solutions and buffers for ELISAs

Coating buffer, pH 9.6 (For IL-8, RANTES, and MCP-1)

15mM Na_2CO_3

35mM $NaHCO_3$

3mM NaN_3

Adjust pH to 9.6 with 1 M HCl. Buffer can be kept for 2 weeks.

Wash buffer, pH 7.3 (For IL-8 and RANTES)

140 mM NaCl

2.7mM KCl

1.5mM KH_2HPO_4

8.1mM Na_2HPO_4

0.05% (v/v) Tween-20

Tris Buffered Saline (TBS), pH 7.5 (For MCP-1)

50mM Tris

150mM NaCl

Adjust pH to 7.5 with HCl.

Wash buffer, pH 7.5 (For MCP-1)

0.05% Tween-20 in TBS

Diethanolamine substrate buffer, pH 9.8 (For IL-8)

10% (v/v) diethanolamine (BDH)

1mM $MgCl_2$

Store in the dark, at 4^0 C.

0.1M Citric-phosphate substrate buffer, pH 5 (For RANTES)

34mM citric acid

66mM Na_2HPO_4

Substrate buffer, pH 9.7 (For MCP-1)

1M Tris

300mM $MgCl_2.6H_2O$

Adjust pH to 9.7 with 1N HCl.

Substrate solution (For MCP-1)

Sigma 104 Phosphate Substrate tablets	5mg (1 tabl)
Substrate buffer	5ml

2.11.4 Solutions and buffers for Northern blot analysis

DEPC-treated water or buffer

Milli-Q water or buffer were treated with 1 ml of diethyl pyrocarbonate (DEPC, Sigma) per litre of water, and incubated overnight at 37^0 C. Autoclave (for 20 minutes at 121^0 C).

20 % (w/v) SDS solution

20 g SDS diluted in 100 ml of sterile Milli-Q water

0.75M Sodium citrate solution, pH 7.0

0.75M trisodium citrate dihydrate in Milli-Q water. Autoclave.

2M TRIS-HCl stock solution, pH 8

2M TRIS in Milli-Q water. Autoclave.

0.5M EDTA stock solution, pH 8

0.5M EDTA in Milli-Q water. Autoclave.

Nucleic acid extraction buffer

4 M Guanidine thiocynate (Fulka, Gillingham, UK) (236.32 g / 500 ml)

25 mM Sodium citrate (17.6 ml of 0.75 M stock / 500 ml)

0.5 % sarcosyl (8.3 ml of 30% stock (BDH)/500 ml)

in Milli-Q water.

Add 0.1M 2-mercaptoethanol (Sigma) prior to use.

Use buffer containing 2-mercaptoethanol within 1 month. Store at 4^0 C.

Phenol extraction buffer

100 mM Tris (25ml of 2M stock/500ml)

10mM EDTA (10ml/ of 0.5M stock/500ml)

1% SDS (25ml of 20% stock/500ml)

in Milli-Q water. Autoclave.

3M Sodium acetate, pH 5.2

3M sodium acetate dissolved in 250 ml Milli-Q water.

Adjust pH to 5.2 using 3M glacial acid. DEPC-treat and autoclave.

75% (v/v) ethanol solution

75 ml absolute ethanol (Hayman Ltd, Witham, UK) in 25 ml DEPC-treated water.

20X MOPS running buffer

0.4M 3-[N-morpholino]-propane-sulfonic acid (MOPS)

0.02M EDTA (4ml of 0.5M stock/100ml)

0.2M sodium acetate (6.64ml of 3M stock/100ml)

Adjust volume to 100ml with Milli-Q water and pH with solid NaOH. Filter sterilise.

1X MOPS running buffer

50ml 20X MOPS in 950ml DEPC-treated water.

1mg/ml Ethidium bromide solution

10mg ethidium bromide (Sigma) in 10 ml DEPC-treated water.

RNA Sample buffer (per sample)

7µl 36 % (w/v) formaldehyde (BDH)

4µl 20X MOPS running buffer

2µl 1mg/ml ethidium bromide solution

20µl formamide (mol. biol. grade, BDH)

Made up fresh immediately before use.

Bromophenol solution

0.025g bromophenol blue

3ml glycerol

Adjust volume to 10ml with DEPC-treated water.

20x SSC, pH 7

3M NaCl

0.3M trisodium citrate dihydrate

in Milli-Q water. DEPC-treat and autoclave.

Buffer 1

0.1M maleic acid

0.15M NaCl

Adjust to pH 7.5 with solid NaOH. DEPC-treat and autoclave.

Blocking stock solution

10g blocking reagent (Boerhinger Mannheim, Lewes, UK) in 100ml buffer 1.

Microwave to dissolve (do not boil). Autoclave.

Hybridisation solution

5X SSC	(25ml of 20X stock/100ml)
0.1% sarcosyl	(0.33ml of 30% stock/100ml)
0.02% SDS	(0.1ml of 20% stock/100ml)
1% blocking buffer	(10ml of 10% stock/100ml)

in DEPC-treated water.

2X SSC, 0.1% SDS solution

50ml of 20X SSC

2.5ml of 20% SDS stock

Adjust volume to 500ml with DEPC-treated water.

0.1X SSC, 0.1% SDS solution

2.5ml of 20X SSC

2.5ml of 20% SDS stock

Adjust volume to 500ml with DEPC-treated water.

Washing buffer

0.3% (v/v) Tween-20 (Sigma) in buffer 1.

Buffer 2

10% (v/v) blocking stock solution in buffer 1.

Buffer 3, pH 9.5

0.1M TRIS

0.1M NaCl

in sterile Milli-Q water.

2.11.5 Solutions and buffers for SDS-PAGE and Western blotting

1M TRIS-HCl, pH 8.8

1M TRIS

in Milli-Q water.

1M TRIS-HCl, pH 6.8

1M TRIS

in Milli-Q water.

10% (w/v) SDS solution

5g of SDS in 50ml Milli-Q water.

10% (w/v) Ammonium Persulphate Solution (APS)

0.1g ammonium persulphate (Sigma) in 1ml Milli-Q water.

Made up fresh on day.

1X Sample buffer

10% SDS	5ml
glycerol	5ml
1M Tris pH 6.8	4ml
Mercaptoethanol (Sigma)	2.5ml
100mM EDTA	9ml
H_2O	19.5ml
Bromophenol blue (Bio-Rad)	0.01g

7 % (w/v) acrylamide running gel:

Protogel {30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide}	3.5 ml
1M Tris-HCl, pH 5.6	5.6 ml
10 % SDS solution	0.15 ml
10 % ammonium persulphate (APS)	0.1 ml
Double-distilled water	5.85 ml
N,N,N',N',tetramethylene-diamine (TEMED) (Sigma)	10 µl

5 % (w/v) acrylamide stacking gel:

Protogel {30 % (w/v) acrylamide, 0.8 % (w/v) bisacrylamide}	1.67 ml
1M Tris-HCl, pH 5,6	1.25 ml
10 % SDS solution	0.1 ml
10 % ammonium persulphate (APS)	0.05 ml
Double-distilled water	6 ml
N,N,N',N',tetramethylene-diamine (TEMED) (Sigma)	10 µl

Electrophoresis buffer

25mM TRIS

192mM glycine

0.1% SDS

Transfer buffer

25mM TRIS

192mM glycine

20% methanol

Coomassie blue stain

40% (v/v) methanol

7% (v/v) acetic acid

0.1% (w/v) coomassie brilliant blue R-250 (Sigma)

in Milli-Q water.

Destain solution

40% (v/v) methanol

7% (v/v) acetic acid

in Milli-Q water.

2.11.6 Solutions and buffers for Immunohistochemistry

Blocking reagent

H_2O_2	5ml
Methanol	200ml

Tris Buffered saline (TBS), pH 7.6

50mM Tris

150mM NaCl

Adjust pH to 7.6 with HCl.

Normal Rabbit Serum 5% (For monoclonals)

TBS, pH 7.6	200ml
NaN_3	0.2 g
Add normal Rabbit serum	10 ml

Normal Swine Serum 5% (For polyclonals)

TBS, pH 7.6	200ml
NaN_3	0.2 g
Add normal Swine serum	10 ml

Avidin-Biotin Complex (ABC)

TBS, pH 7.6	1ml
Reagent A	10µl
Reagent B	10µl

DAB Reagent

3.3' Diaminobenzidine (DAB)	0.1 g
H_2O_2	200µl
Distilled water	200ml

Haematoxylin (Gills) 50%

Gills HX	200ml
Distilled water	200ml

3. RESULTS

3.1 INDUCIBLE NITRIC OXIDE EXPRESSION AND ACTIVITY BY COLONIC EPITHELIAL CELLS

3.1.1 Nitrite generation by HT-29 cells

Growth arrested monolayers of HT-29 cells when stimulated with vehicle produced a small constitutive amount of nitrite (Fig. 2). Time course studies revealed a time-dependent increase in constitutive nitrite generation of 50 ± 4, 75 ± 8, and 103 ± 8 nM/10^6 cells (n=3) at 24h, 48h, and 72h, respectively (Fig. 3). The pro-inflammatory cytokines IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml), added alone to HT-29 cells did not induce a significant increase in nitrite generation compared to the constitutive nitrite production in vehicle treated cells (Fig. 2). The combination IL-1α/IFN-γ was the minimal requirement for enhanced nitrite production, while other pairs of cytokines were ineffective. Stimulation with IL-1α (10ng/ml)/IFN-γ (300U/ml) produced a highly significant ($p < 0.001$) increase in nitrite production of 306 ± 24 nM/10^6 cells (n=3) at 48 hours, compared to the basal production of 75 $\pm$ 8 nM/10^6 cells (n=3) (Fig. 2). The addition of TNF-α to the combination of IL-1α/IFN-γ produced approximately a three fold enhancement of IL-1α/IFN-γ-induced nitrite generation eg 889 ± 35 nM/10^6 cells (n=3) compared to 306 ± 24nM/10^6 cells (n=3) at 48h (Fig 2).

Stimulation of HT-29 cells with IL-1α (10ng/ml)/IFN-γ (300U/ml) induced a time-dependent generation of nitrite at 24 (153 ± 7), 48 (306 ± 24) and 72 (384 ± 15) hours compared to basal

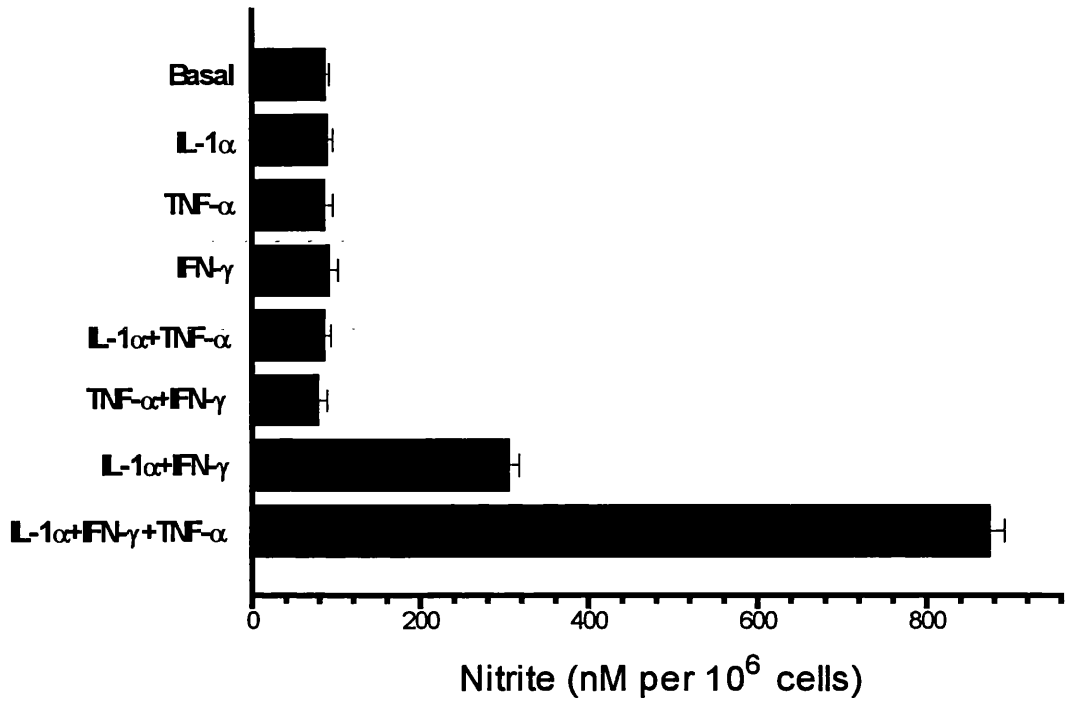

Figure 2 Nitrite production by HT-29 colonic epithelial cells following stimulation for 48 hours incubation at 37° C with vehicle, IL-1α (10ng/ml), TNF-α (100ng/ml), and IFN-γ (300U/ml) added alone or in combination. Nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. Basal is the amount of nitrite produced by HT-29 cells in the absence of added cytokines. Each bar is the mean ± SE of three experiments.

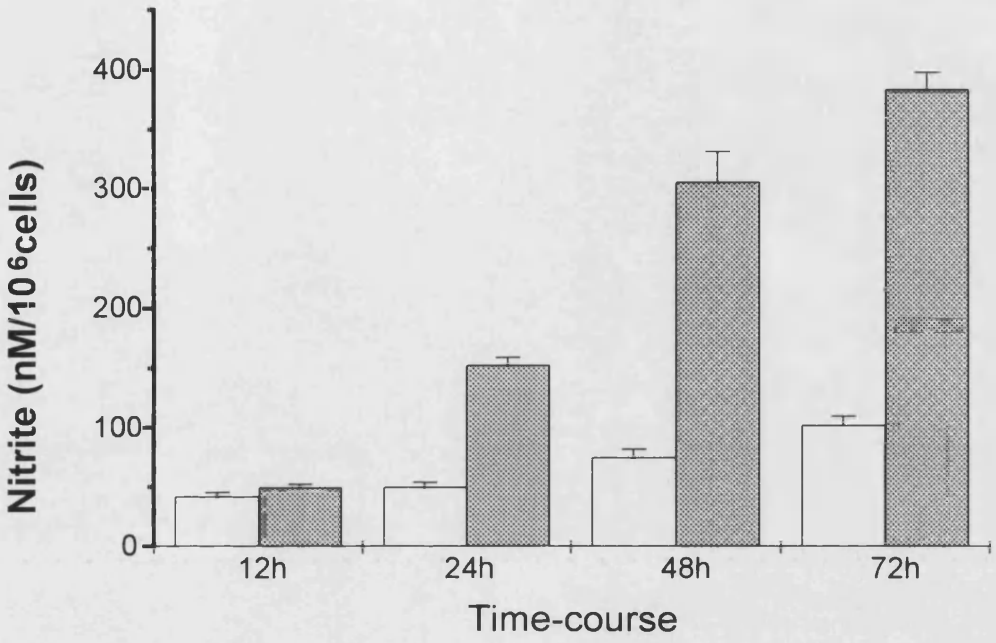

Figure 3 Time course of nitrite production by HT-29 cells following stimulation with vehicle (open bars) or IL-1α (10ng/ml)/IFN-γ (300U/ml) (solid bars). Nitrite levels were determined in supernatants, after incubation at 37° C, using a fluorescent substrate with a 10 nM level of detection. Each bar is the mean ± SE of three experiments.

values of 50 ± 4, 75 ± 8, and 103 ± 8 nM/10^6 cells respectively (n=3) (Fig. 3). Stimulation with increasing concentrations of IL-1α (0-10ng/ml) in the presence of IFN-γ (300U/ml) was found to produce a concentration dependent generation of nitrite production at 48 hours by HT-29 cells (Fig. 4a). Similarly, increasing concentrations of IFN-γ (0-300U/ml) in the presence of IL-1α (10ng/ml) were found to produce a concentration related production of nitrite at 48 hours (Fig. 4b). Interestingly different concentrations of TNF-α(0-100ng/ml) in the presence of the combination IL-1α(10ng/ml)/IFN-γ(300U/ml) induced a concentration dependent enhancement of nitrite production (Fig. 5). Pre-treatment of HT-29 colonic epithelial cells with the protein synthesis inhibitor cycloheximide (5 mg/ml) for 1 hour prior to IL-1α(10ng/ml)/IFN-γ (300U/ml) stimulation reduced the nitrite levels from 306 ± 24 to 80 ± 6 nM/10^6 cells (n=3) at 48 hours, which was no different to basal nitrite production of 75 ± 8 nM/10^6cells. Finally, treatment of unstimulated cells with cycloheximide (5mg/ml) did not affect the basal nitrite production (74 ± 4 vs 75 ± 8 nM/10^6cells).

To examine the possible modulatory effect of IL-13, IL-4, and IL-10 on the cytokine-induced nitrite generation confluent monolayers of HT-29 cells, after 1h pre-treatment with various concentrations (0.1-30ng/ml) of IL-13, IL-4 or IL-10 were stimulated with either IL-1α (10ng/ml)/IFN-γ (300U/ml) or IL-1α (10ng/ml)/IFN-γ (300U/ml)/TNF-α (100ng/ml) supernatants were collected and nitrite were determined at 24 and 48 hours. The stimulation of HT-29 cells with IL-13, IL-4, and IL-10, added alone, up to 72 hours did not affect the constitutive nitrite generation by these cells (Fig 6). At the stimulation with IL-1α (10ng/ml)/IFN-γ (300U/ml)/TNF-α (100ng/ml) IL-13 (0.3-30ng/ml) and IL-4 (0.3- 30ng/ml) produced a significant suppression of nitrite generation ($p < 0.01$ - $p < 0.001$) (Fig 7a,8a). The same concentration range of IL-13 and IL-4 had a different effect on IL-1α/IFN-γ-induced

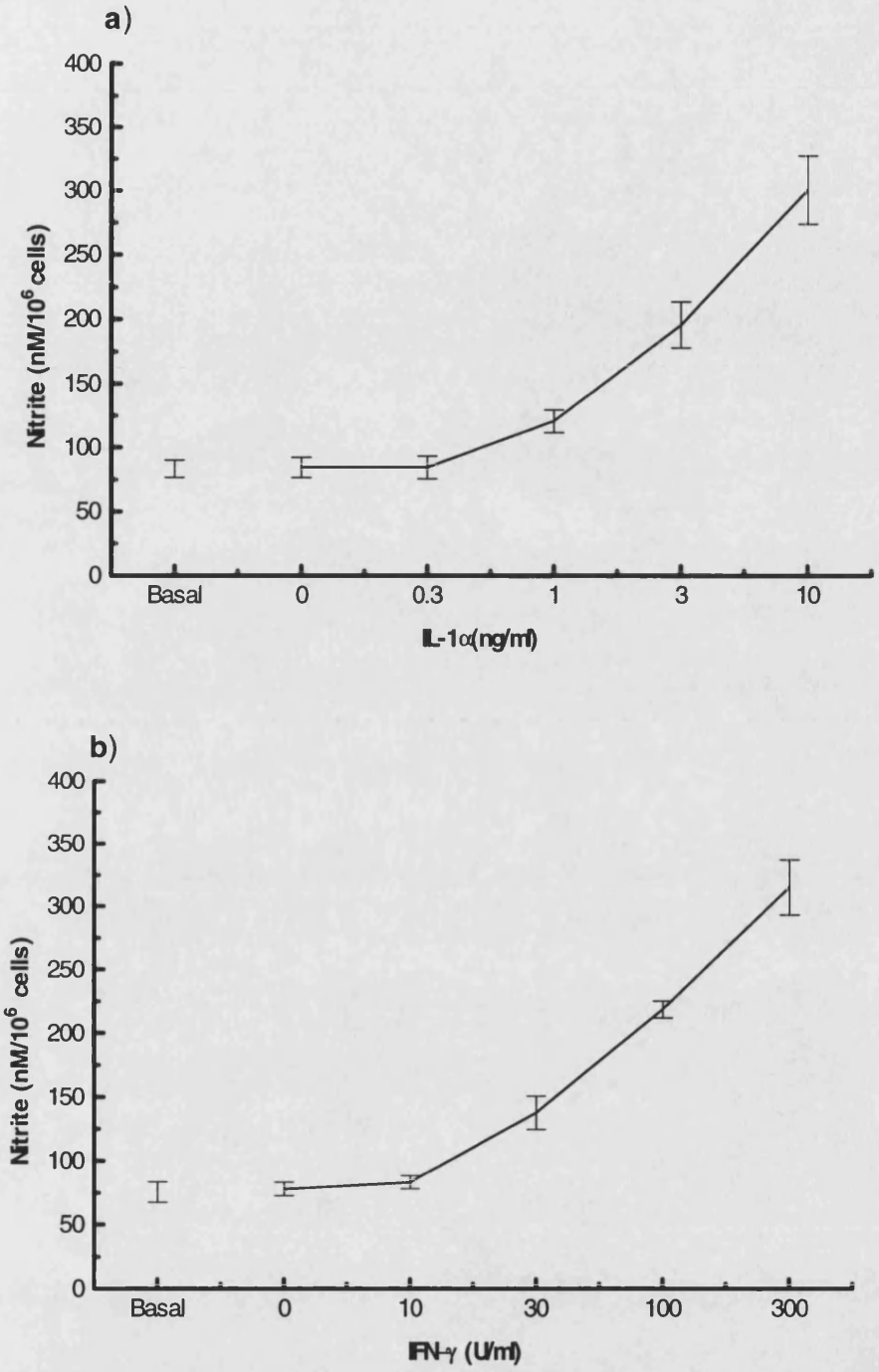

Figure 4. a) Nitrite production by HT-29 cells after 48h treatment with different concentrations of IL-1α (0-10ng/ml) in the presence of IFN-γ (300U/ml). b) Nitrite production by HT-29 cells after 48h treatment with different concentrations of IFN-γ (0-300U/ml) in the presence of IL-1α (10ng/ml). Each point is the mean ± SEM of three experiments.

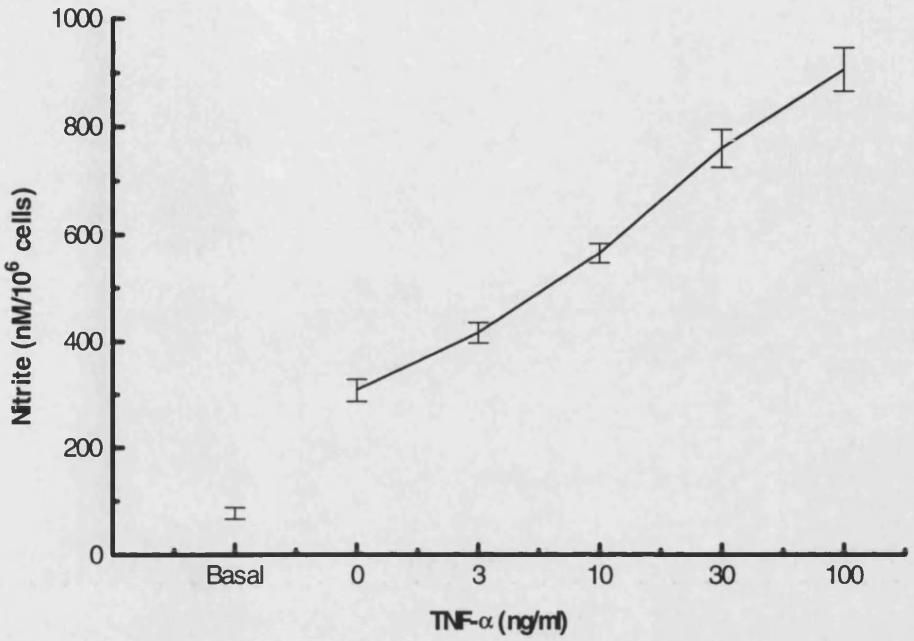

Figure 5. Effect of TNF-α (0-100ng/ml) on IL-1α (10ng/ml)/IFN-γ (300U/ml)-induced nitrite generation after 48h treatment by HT-29 cells. Nitrite levels were determined in supernatants, after incubation at 37° C, using a fluorescent substrate with a 10 nM level of detection. Basal is the amount of nitrite produced by HT-29 cells in the absence of added cytokines. Each point is the mean ± SEM of three experiments.

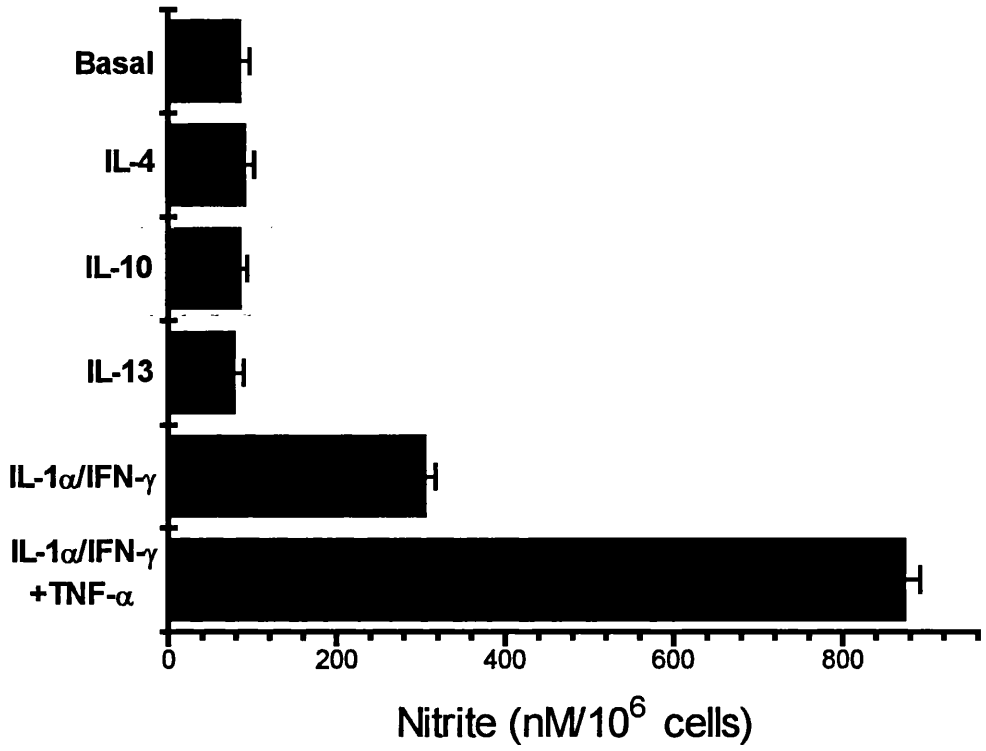

Figure 6 Nitrite production by HT-29 cells after treatment with cytokines. Confluent monolayers of HT-29 cells were treated with IL-13 (10ng/ml), IL-4 (10ng/ml), IL-10 (10ng/ml), IL-1α (10ng/ml)/IFN-γ (300U/ml) or IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) and after 48 hours incubation at 37° C nitrite levels were determined in supernatants, using a fluorescent substrate with a 10 nM level of detection. Basal is the amount of nitrite produced by HT-29 cells in the absence of added cytokines. The result represent the mean ± SEM of the three separate experiments.

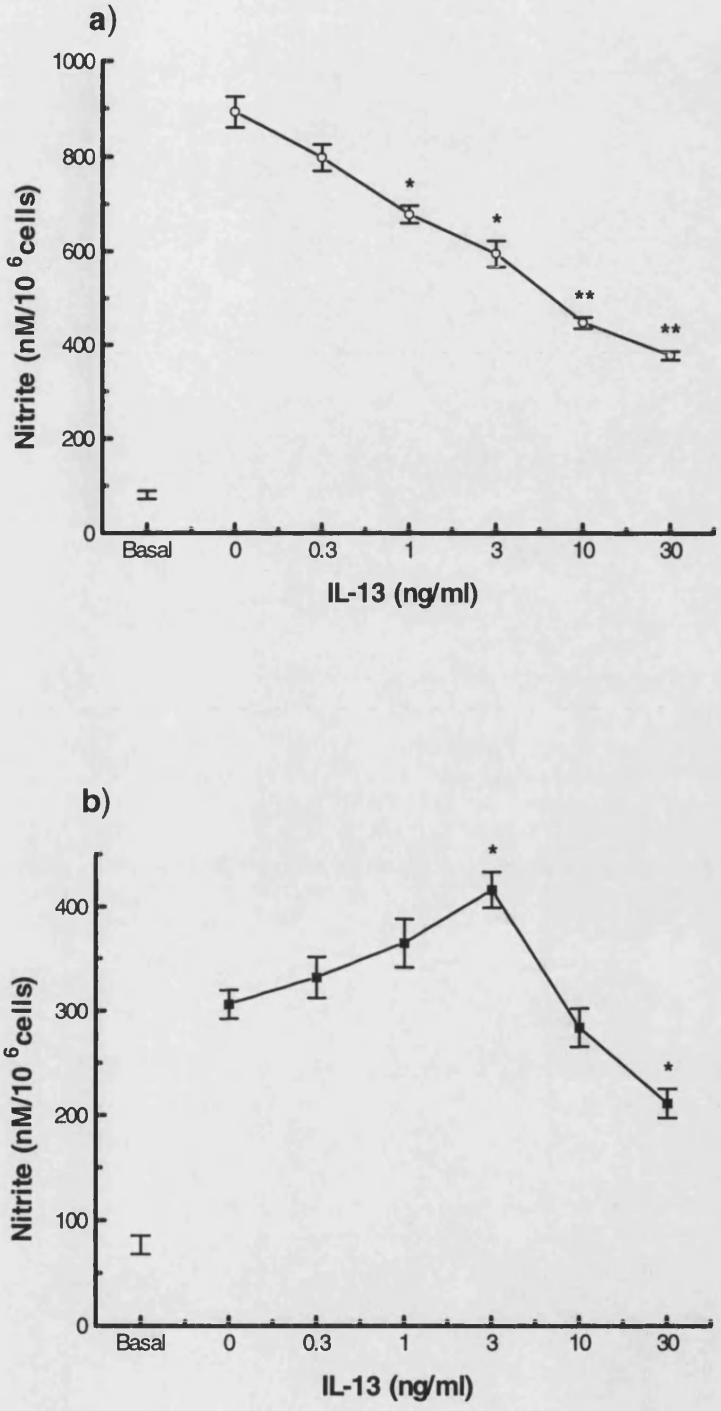

Figure 7. Nitrite production by HT-29 cells after treatment with (a) IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) or (b) IL-1α(10ng/ml)/IFN-γ(300U/ml) in the presence of increasing concentrations of IL-13. Each point represent the mean ± SEM of three separate experiments (* $p < 0.01$, ** $p < 0.001$), compared with positive control (0) response.

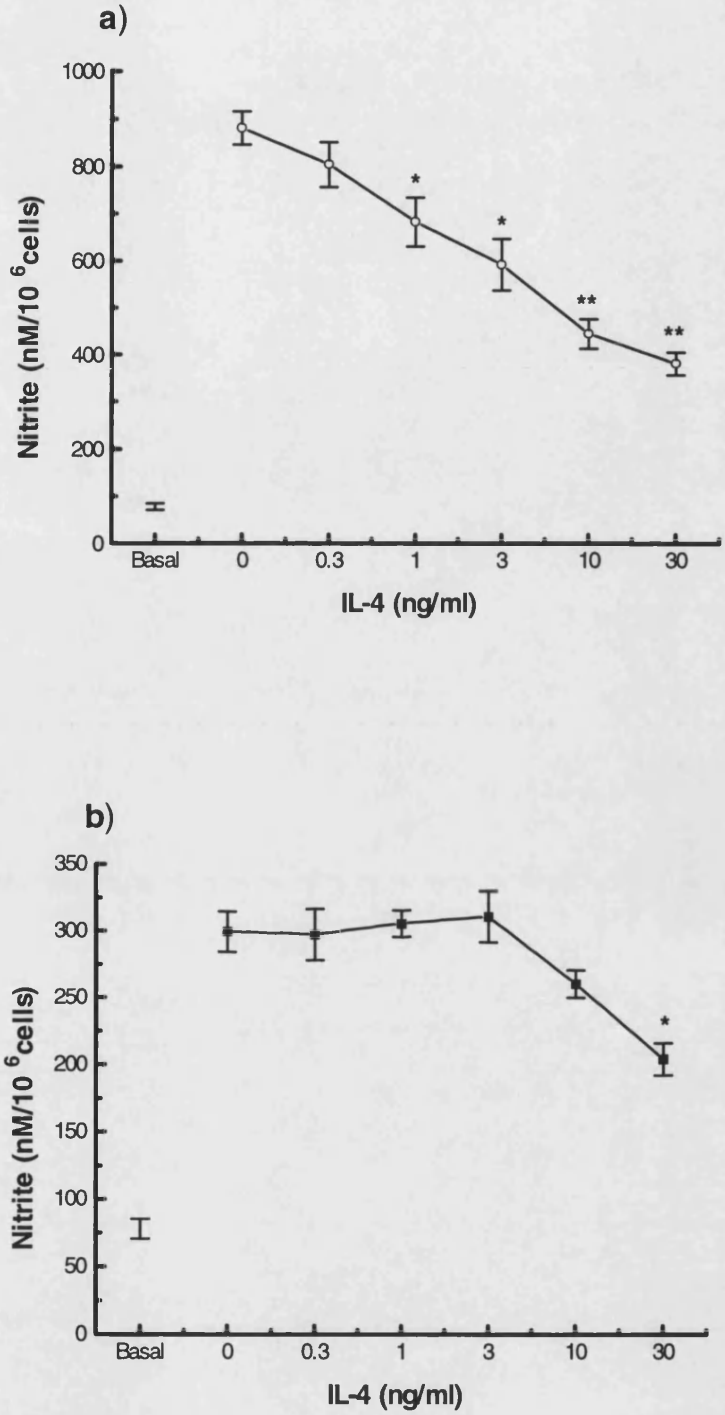

Figure 8. Nitrite production by HT-29 after treatment with (a) IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) or (b) IL-1α(10ng/ml)/IFN-γ(300U/ml) in the presence of increasing concentrations of IL-4. Each point represent the mean ± SEM of three separate experiments (* $p < 0.01$, * $p < 0.001$), compared with positive control (0) response.

nitrite generation. Low concentrations (0.3-3ng/ml) of IL-13 but not IL-4 produced a significant enhancement ($p < 0.01$), while doses of IL-13 and IL-4 higher than 10ng/ml suppressed significantly ($p < 0.01$ at 30ng/ml) the nitrite generation by HT-29 cells (Fig. 7b,8b). In marked contrast the same concentration range of IL-10 had no effect on IL-1α/IFN-γ (Fig 9a) or on IL-1α/IFN-γ/TNF-α-induced nitrite generation by HT-29 cells (Fig 9b).

3.1.2 Inducible NOS mRNA expression by HT-29 cells

To determine whether the inducible generation of nitric oxide by HT-29 epithelial cells was due to the induction of iNOS, the iNOS mRNA expression was determined by Northern blot analysis. The time course of iNOS mRNA expression in epithelial cells after stimulation with IL-1α(10ng/ml)/IFN-γ(300U/ml) was examined. In unstimulated cells iNOS transcripts were not detected at any of the time points. The combination IL-1α (10ng/ml)/IFN-γ(300U/ml) induced iNOS mRNA expression, which was first observed at 6h, peaked at 24h and was undetectable by 72h (Fig. 10). Increasing concentrations of IL-1α(0-10ng/ml) in the presence of IFN-γ(300U/ml) were found to produce concentration related iNOS mRNA expression after 24h treatment (Fig. 11a). Similarly, using different concentrations of IFN-γ(0-300U/ml) in the presence of IL-1α(10ng/ml) we found concentration dependent iNOS mRNA expression at 24 hours in HT-29 cells (Fig. 11b). These results demonstrate that both IL-1α and IFN-γ produce concentration dependent iNOS mRNA expression in HT-29 cells which induces concentration dependent nitrite formation by these cells(Fig. 4a, 4b). In marked contrast, experiments using different concentrations of TNF-α(3-100ng/ml) in the presence of IL-1α (10ng/ml)/IFN-γ(300U/ml) demonstrate that the addition of TNF-α was without effect on the IL-1α/IFN-γ-induced

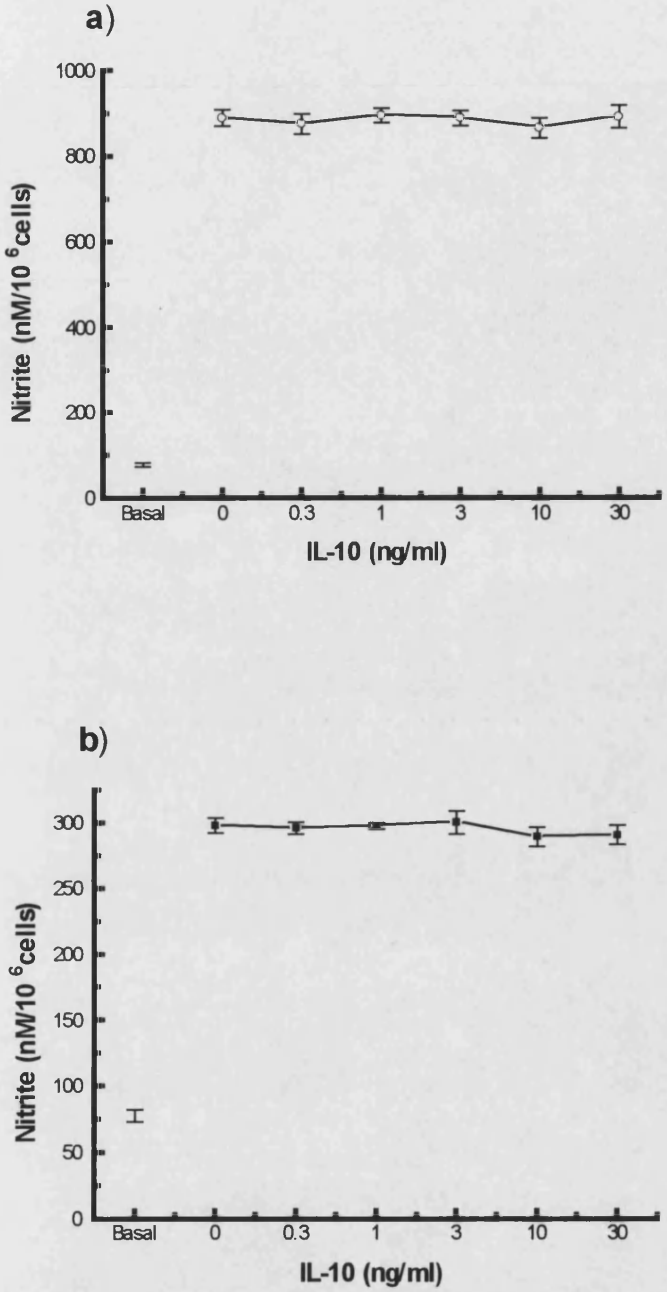

Figure 9 Nitrite production by HT-29 cells after treatment with (a) IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) or (b) IL-1α(10ng/ml)/IFN-γ(300U/ml) in the presence of increasing concentrations of IL-10. Each point represent the mean ± SEM of three separate experiments.

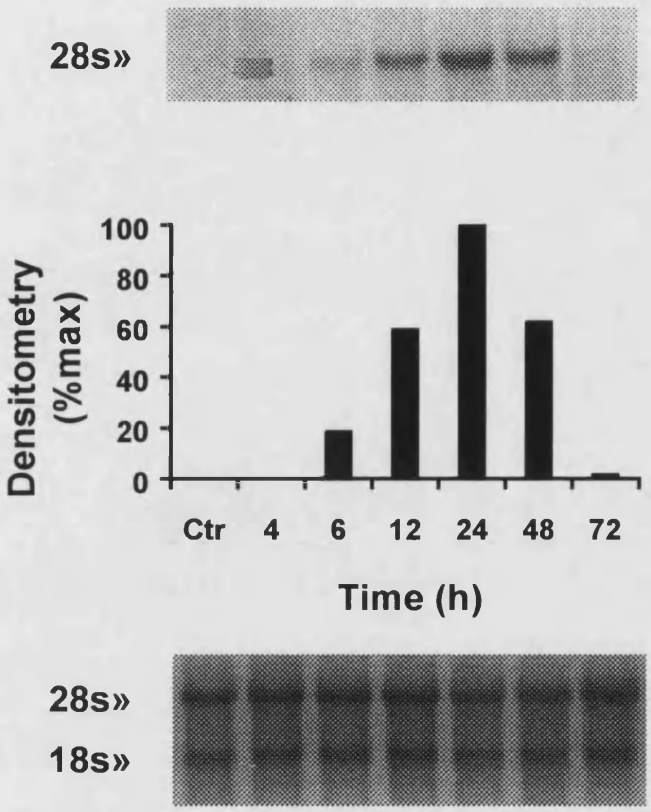

Figure 10 Northern blot analysis of time course of iNOS mRNA expression in HT-29 cells stimulated with IL-1α (10ng/ml)/IFN-γ (300U/ml). The top panel is the northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

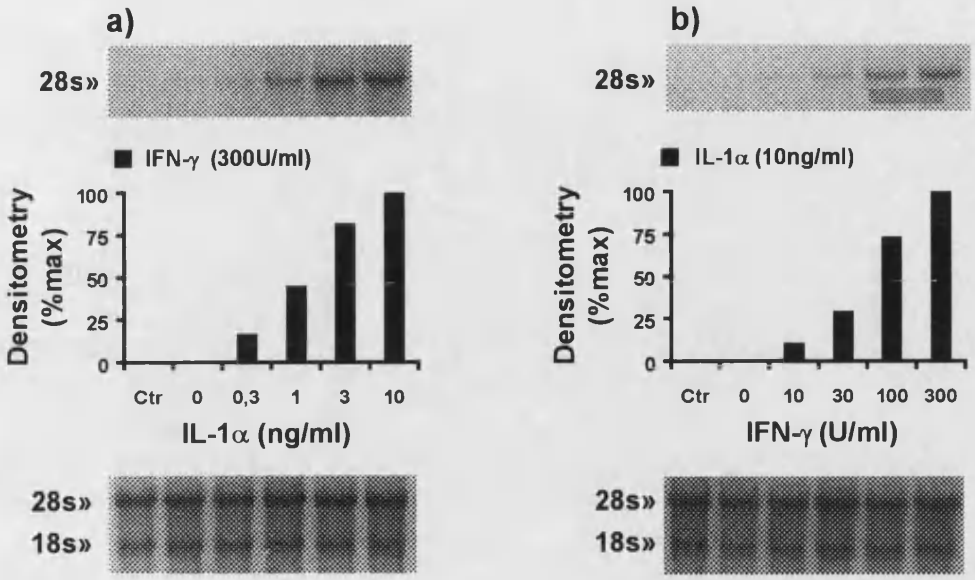

Figure 11 a) iNOS mRNA expression in HT-29 cells after 24h treatment with different concentrations of IL-1α (0-10ng/ml) in the presence of IFN-γ (300U/ml). b) iNOS mRNA expression in HT-29 cells after 24h treatment with different concentrations of IFN-γ (0-300U/ml) in the presence of IL-1α (10ng/ml). The top panel of each figure is the northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of two experiments.

iNOS expression in HT-29 colonic epithelial cells (Fig. 12). These results suggest that the up-regulation by TNF-α of the IL-1α/IFN-γ-induced nitrite generation by HT-29 cells is at the post-transcriptional level.

To determine the effect of IL-13, IL-4 and IL-10 on the cytokine-induced iNOS mRNA expression, cells were pretreated for 1h with different doses (0.3-30ng/ml) of IL-13, IL-4 or IL-10, then IL-1α (10ng/ml)/ IFN-γ (300U/ml) or IL-1α (10ng/ml)/ IFN-γ (300U/ml)/ TNF-α (100ng/ml) were added and iNOS mRNA expression was measured at 24h. Low doses (0.3-3ng/ml) of IL-13 but not of IL-4 were found to increase the IL-1α/IFN-γ-induced iNOS mRNA expression, while high doses (10-30ng/ml) of both IL-13 and IL-4 suppressed significantly the iNOS mRNA expression by HT-29 cells, in the same way as they regulated the nitrite generation (Fig. 13a,14a). Similarly, low doses (0.3-3ng/ml) of IL-13 but not IL-4 were found to increase the IL-1α/IFN-γ/TNF-α-induced iNOS mRNA expression, while higher doses of both IL-13 (Fig. 13b) and IL-4 suppressed significantly the iNOS mRNA expression by HT-29 cells, likewise they regulated the IL-1α/IFN-γ-induced iNOS mRNA expression, and in marked contrast with the regulation by IL-13 and IL-4 of the IL-1α/IFN-γ/TNF-α -induced nitrite generation. These data taken together suggest that IL-13 and IL-4, at all concentrations they were examined, down-regulate the post-transcriptional effect of TNF-α on the IL-1α/IFN-γ-induced nitrite generation by HT-29 cells, while high concentrations (>10 ng/ml) in addition blocked iNOS mRNA induced by proinflammatory cytokines.

Finally, pre-treatment with IL-10 had no effect on the cytokine-induced mRNA in HT-29 cells (Fig. 14b).

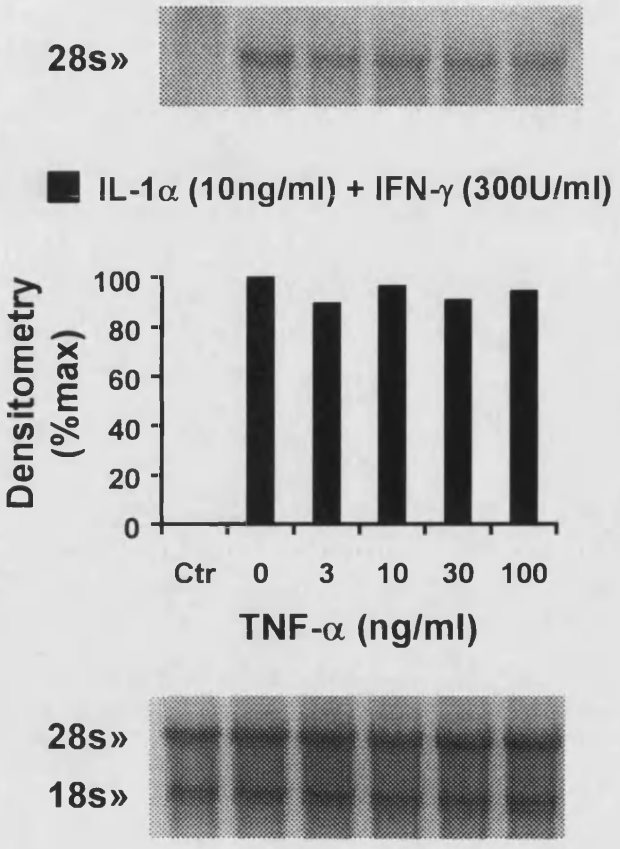

Figure 12 Effect of TNF-α (0-100ng/ml) on IL-1α (10ng/ml)/IFN-γ (300U/ml)-induced iNOS mRNA expression in HT-29 cells after 24h treatment. The top panel is the northern blot, the middle panel is the densitometry analysis of blot and the lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

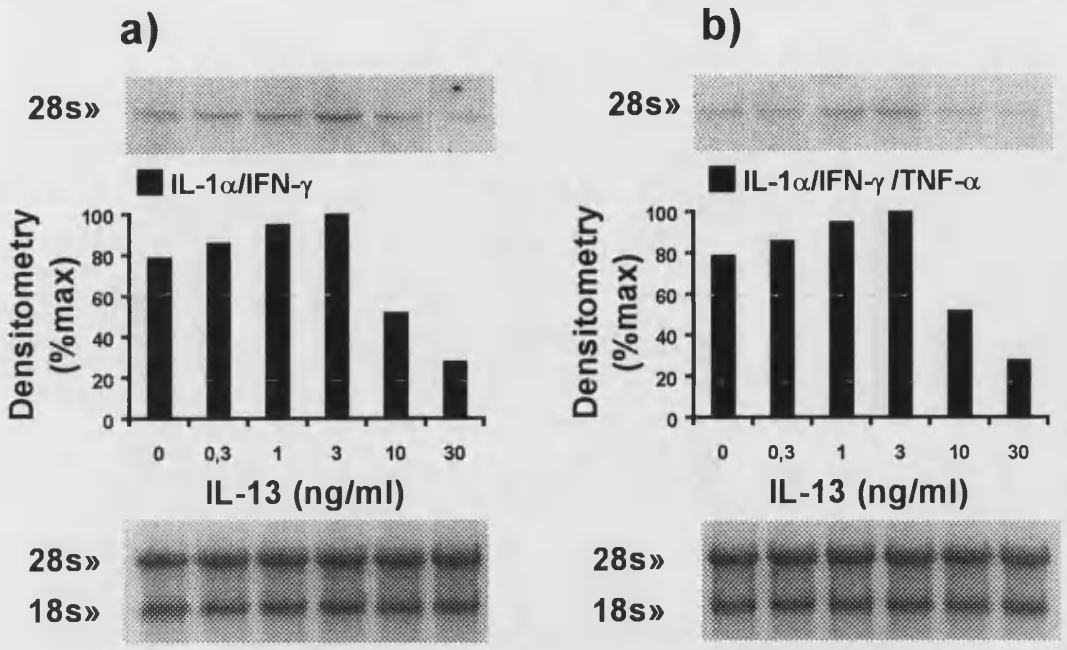

Figure 13 iNOS mRNA expression by HT-29 cells after treatment with (a) IL-1α(10ng/ml)/IFN-γ(300U/ml) or (b) IL-1α(10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) in the presence of increasing concentrations of IL-13. Pro-inflammatory cytokines were added after 1h pretreatment with IL-13 and incubated for 12h at 37° C. The top panel is the Northern blot hybridized at 60° C overnight with DIG-labelled oligonucleotide probes (10ng/ml) for iNOS, the middle panel is the densitometry analysis of blot and the lower panel the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

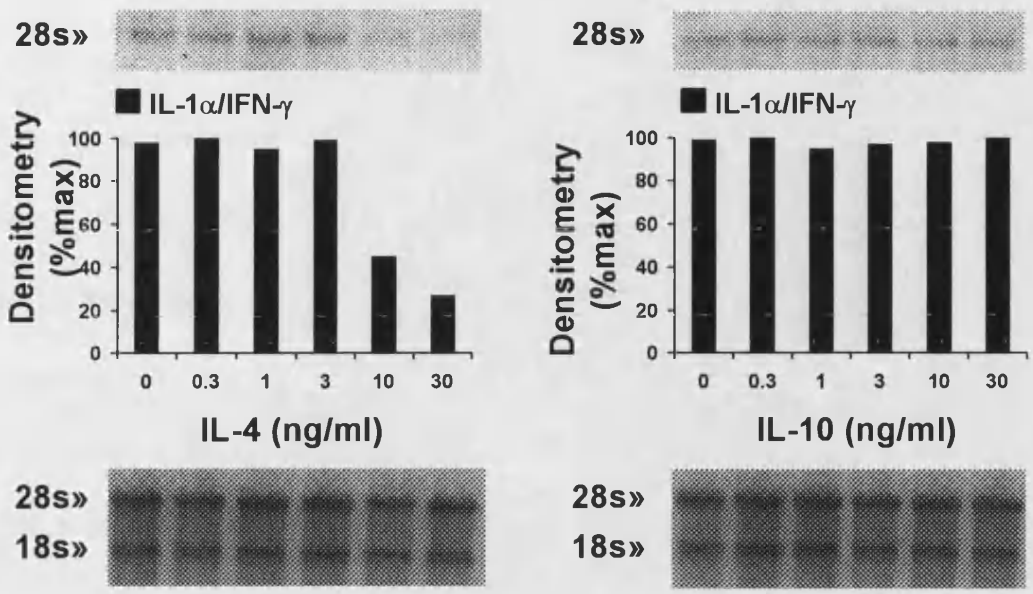

Figure 14 iNOS mRNA expression by HT-29 cells after treatment with IL-1α(10ng/ml)/IFN-γ(300U/ml) in the presence of increasing concentrations of IL-4 (a) or IL-10 (b). Pro-inflammatory cytokines were added after 1h pretreatment with either IL-4 or IL-10 and incubated for 12h at 37° C. The top panel is the Northern blot hybridized at 60° C overnight with DIG-labelled oligonucleotide probes (10ng/ml) for iNOS, The top panel is the Northern blot, the middle panel is the densitometry analysis of blot and the lower panel the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

3.1.3 iNOS protein expression

HT-29 cells were treated for 24 hours with the appropriate stimuli, then the iNOS protein expression was determined by Western analysis. The minimal requirement for iNOS protein expression is the combination of IL-1α/IFN-γ, while the iNOS protein expression is greatly enhanced by the addition of TNF-α, in parallel to the 3 fold increase of nitrite generation. Unstimulated cells did not express iNOS protein. Pre-treatment of HT-29 cells for 1 hour with low concentrations of IL-13 (0.3 - 30 ng/ml) slightly increased, while higher concentrations (10 - 30 ng/ml) suppressed iNOS protein expression induced by the minimal cytokine requirement of IL-1α (10ng/ml)/ IFN-γ (300U/ml) combination (Fig. 15a). In contrast, pretreatment for 1 h with IL-13 (0.3 -30 ng/ml) produced a concentration dependent inhibition of iNOS protein expression induced by the optimal cytokine "cocktail" of IL-1α (10ng/ml)/ IFN-γ (300U/ml)/ TNF-α (100ng/ml) (Fig. 15b).

3.1.4 Nitrite generation in human colonic biopsies

To examine similar effect of cytokines on the nitrite production by human colonic biopsies and whether they can be modulated by IL-13 colonic biopsies were cultured with the same combination of cytokines under similar conditions. IL-13 added alone did not modulate the constitutive production on either HT-29 cells or colonic biopsy cultures. Mucosal biopsy specimens of each patient were placed in three wells of a six-well plate containing 2 ml of culture medium with the appropriate concentrations of stimuli per well. Colonic biopsies from each patient were then cultured for 30 hours in the presence of either a) vehicle, or b) combination of human recombinant pro-inflammatory cytokines IL-1α (10 ng/ml) + TNF-α

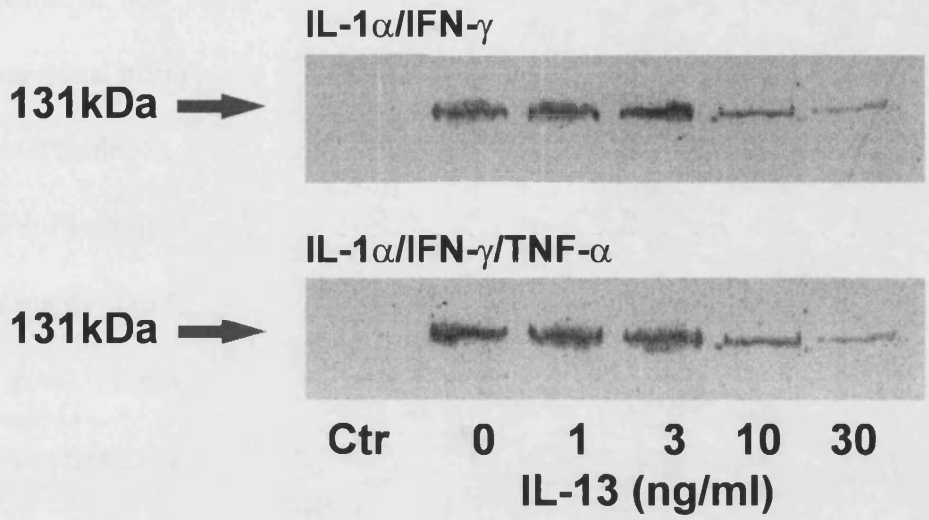

Figure 15. iNOS protein expression by HT-29 cells after stimulation with a): IL-1α (10ng/ml) / IFN-γ (300U/ml) and b): IL-1α (10ng/ml) / IFN-γ (300U/ml) / TNF-α (100ng/ml) in the presence of increasing concentrations of IL-13. HT-29 cells were pretreated for 1h with IL-13, then pro-inflammatory cytokines were added. HT-29 monolayers were scraped after 12h incubation at 37° C and total protein was extracted. iNOS protein expression was determined by Western blot analysis, using an anti-human iNOS antibody. Representative of three experiments.

(100 ng/ml) + IFN-γ (300 U/ml) added after 1 h pre-treatment with vehicle, or c) combination of IL-1α (10 ng/ml) + TNF-α (100 ng/ml) + IFN-γ (300 U/ml) added after 1 hour pre-treatment with IL-13 (30 ng/ml). After treatment, total protein were estimated per well and individual nitrite content of each well was measured and expressed as pmol mg^{-1} of total protein (Table 4). Unstimulated colonic biopsies produced a constitutive amount of nitrite (225 ± 14 pmol/mg of protein of tissue, n=8). Stimulation with IL-1α+TNF-α+IFN-γ produced a highly significant ($p < 0.001$) increase in nitrite production of 784 ± 58 pmol/mg of protein (n=8). Following stimulation with IL-1α+TNF-α+IFN-γ, IL-13 produced a highly significant ($p < 0.001$) suppression of nitrite generation by the cultured colonic biopsies, to 422 ± 39 pmol/mg of protein, at 30ng/ml of IL-13 (n=8) (Fig. 16).

3.1.5 Immunohistochemical study of colonic biopsies

Colonic biopsies of normal, non inflamed mucosa (10 out of 10 cases), derived from patients with diverticular disease, colon adenocarcinoma and healthy individuals were found negative for iNOS expression (Fig. 17A). In 11 out of 12 cases of ulcerative colitis the epithelial cells markedly expressed iNOS always at the superficial part of the crypt and the surface of the mucosa, while no other positive cells were found in lamina propria (Fig. 17B). Characteristically the iNOS expression was located in the most cases at the apical part of epithelial cells and it was in close association with the neutrophil infiltration of lamina propria and epithelium (Fig. 17C). Similar results were seen in all cases of infectious colitis examined (Fig. 17D). Colonic biopsies from infectious colitis patients, positive for iNOS in the acute phase of the disease, when re-examined in total remission did not express detectable iNOS

Patient (No)	Basal	IL-1α/IFN-γ/TNF-α	Presence of IL-13
p1	236	758	314
p2	190	612	384
p3	262	782	348
p4	164	985	462
p5	254	646	516
p6	184	914	390
p7	248	582	326
p8	266	995	638

Table 4. Nitrite production by cultures of human colonic mucosa after stimulation with cytokines. The effect of IL-13. All values are expressed as pmol/mg.

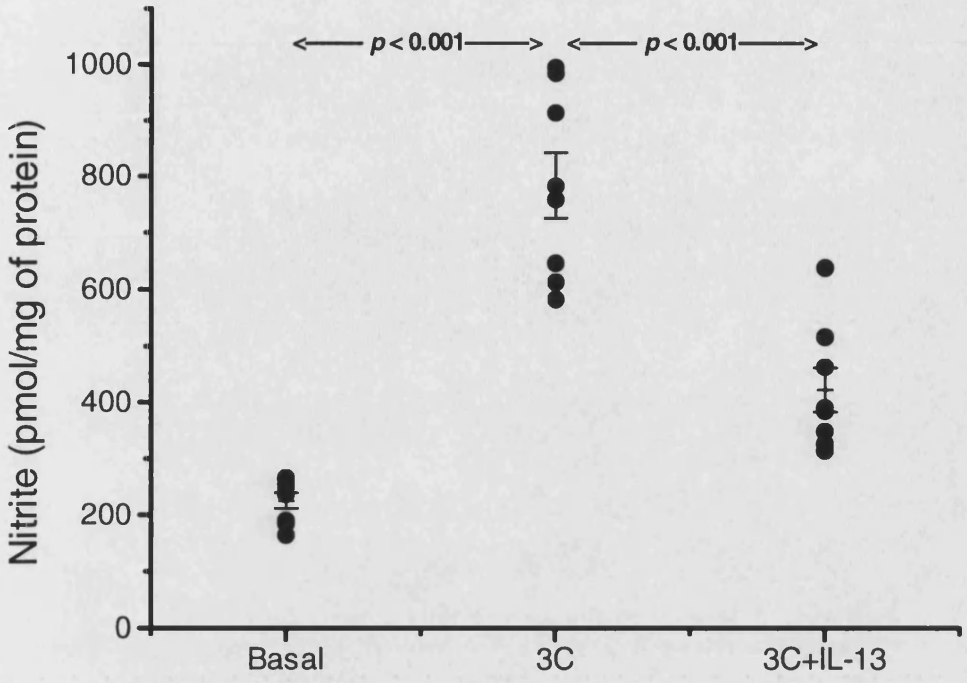

Figure 16. Nitrite production by human colonic biopsies after treatment with (3C), IL-1α (10ng/ml)/IFN-γ(300U/ml)/TNF-α(100ng/ml) in the presence of 30 ng/ml of IL-13. Pro-inflammatory cytokines were added after 1hour pre-treatment with vehicle (3C) or IL-13 (3C+IL-13) and nitrite levels were determined in supernatants after 24h incubation at 37° C. Data are individual datapoint and bars indicate the mean ± SEM of eight samples (n=8).

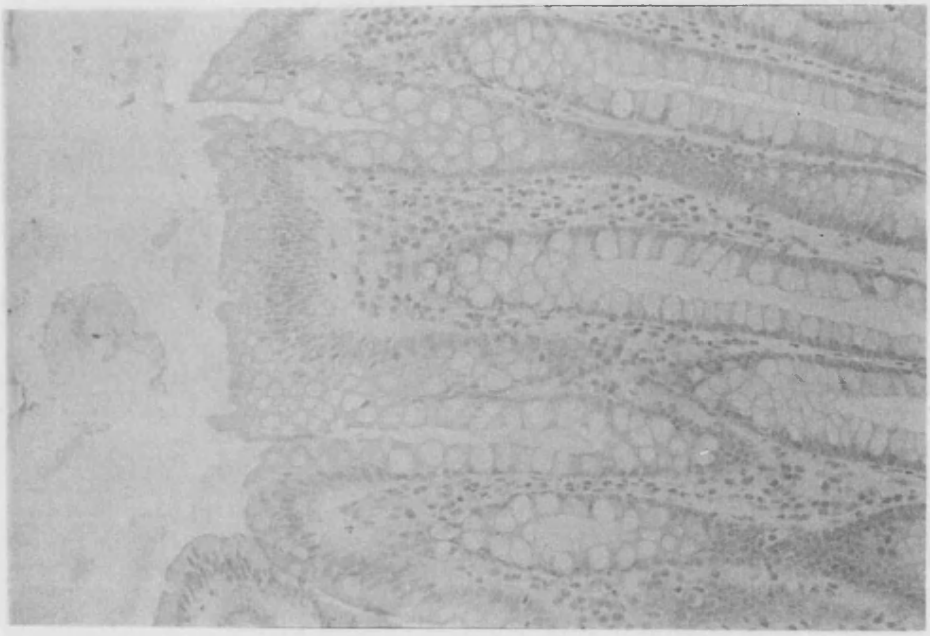

Figure 17A Normal colonic bowel mucosa with no detection of iNOS. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 100.

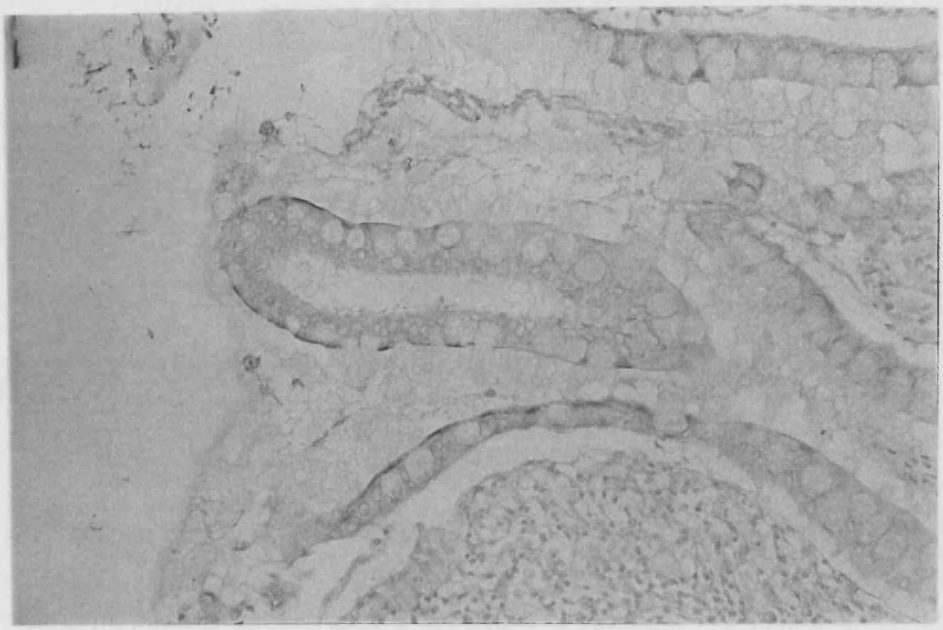

Figure 17B Ulcerative colitis with staining of superficial section of crypt and surface epithelium, while no other positive cells were found in lamina propria. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 100.

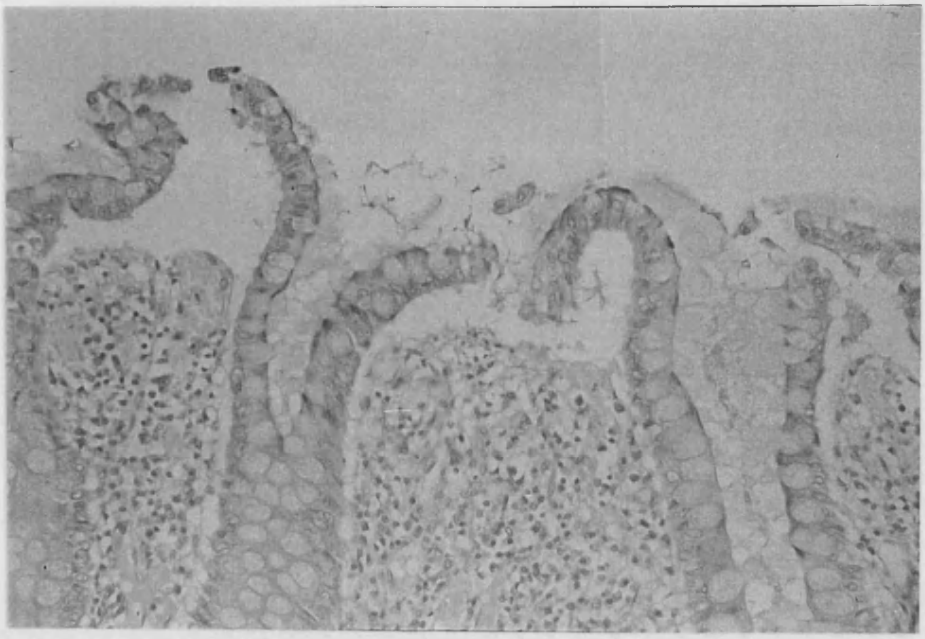

Figure 17C Another ulcerative colitis case showing similar staining; iNOS labelling is located at the apical part of epithelial cells in close association with the neutrophil infiltration of lamina propria and epithelium. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 100.

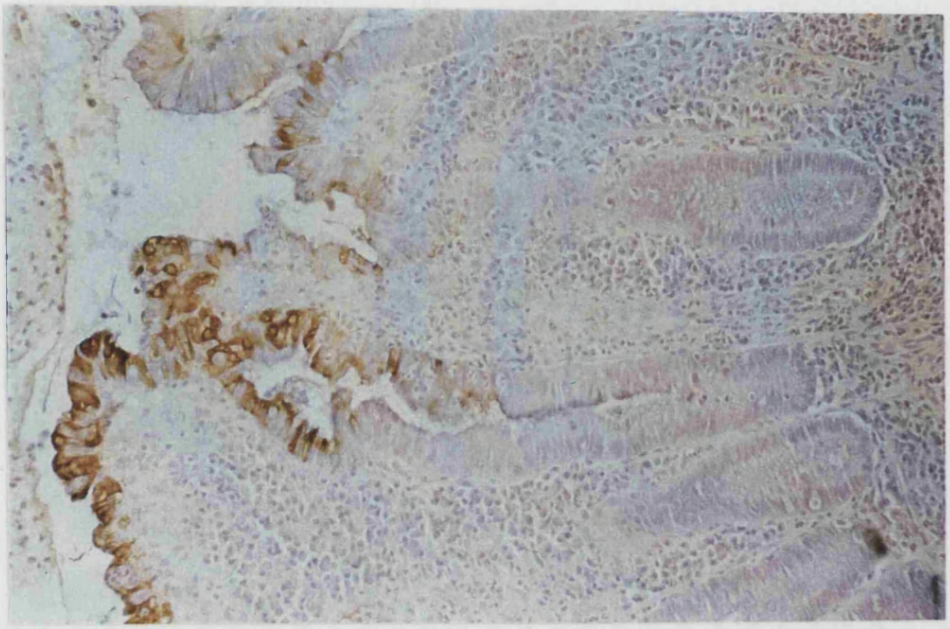

Figure 17D Acute infectious colitis with staining of superficial section of crypt and surface epithelium. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 100.

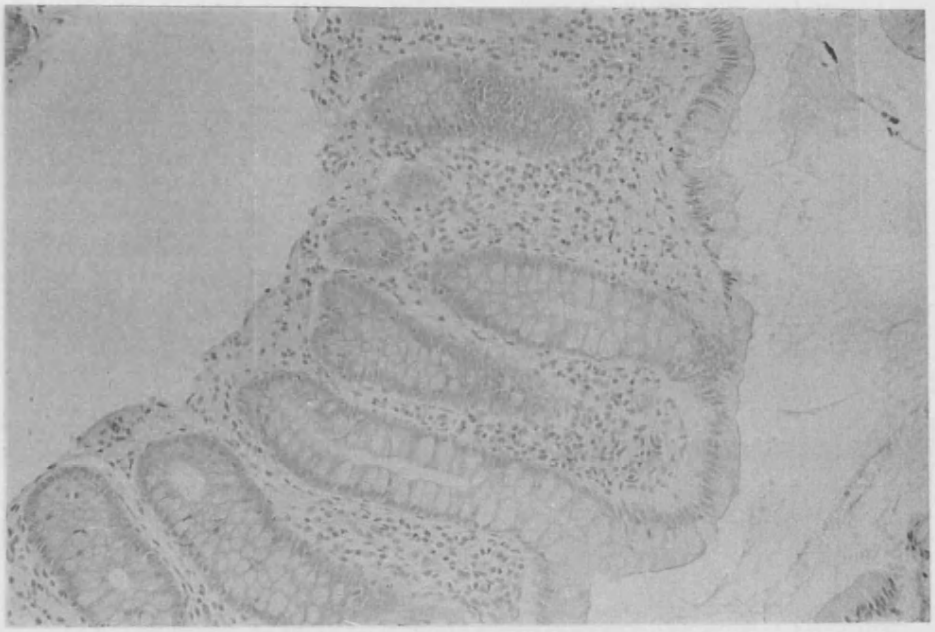

Figure 17E The patient of figure 17D, in total remission, with resolution of iNOS expression. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 50.

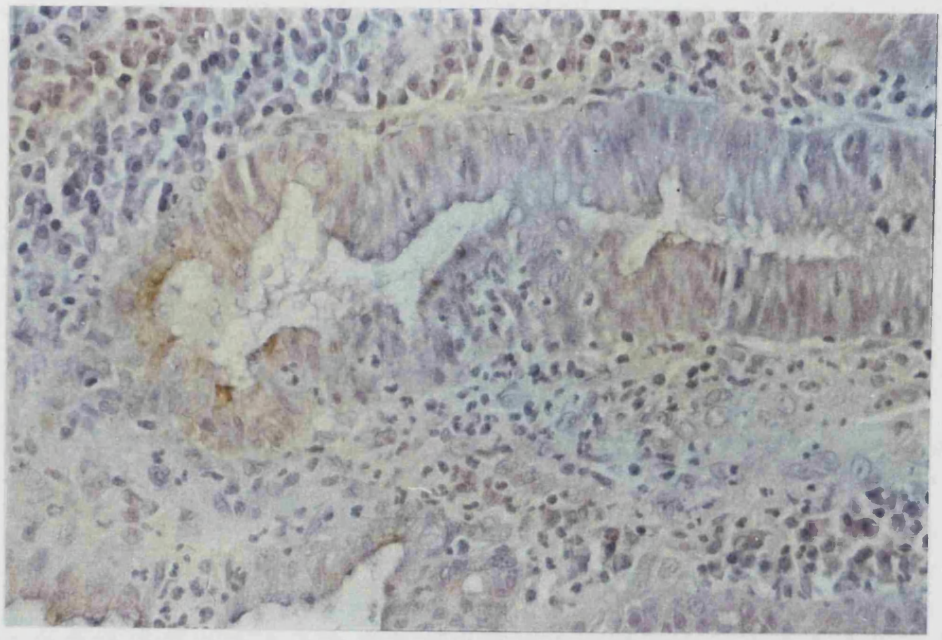

Figure 17F Same section as figure 17D using primary antibody preabsorbed after incubation with the immunogenic peptide. The staining was dramatically reduced. Immunostaining with anti-human iNOS (NO-53) using avidin-biotin peroxidase. Original magnification X 250.

staining (Fig. 17E). No staining was seen in the negative controls, when the primary antibody (NO-53) was omitted, and staining was dramatically reduced when the NO-53 IgG was incubated with the immunogenic peptide (Fig. 17F).

3.1.6 Discussion

NO is produced at many sites of the gastrointestinal tract and it is considered as an important mediator in physiological and pathological events (Whittle, 1994). At the beginning of this study, NO synthesis (Middleton *et al.*, 1993; Lundberg *et al.*, 1994) and NOS activity (Boughton-Smith *et al.*, 1993) had been reported increased in the inflamed mucosa from patients with IBD compared to the uninflamed controls, but the type of cells responsible for this production was unknown. We have examined the source of NO generation in colonic mucosa using immunohistochemical analysis of human large bowel biopsies, cultures of the colonic adenocarcinoma cell line HT-29 and cultures of human colonic biopsies.

We have clearly shown iNOS protein expression via immunohistochemistry in the epithelial cells of the colonic mucosa of patients with active ulcerative colitis and infectious colitis. This iNOS protein expression is absent from the mucosa of biopsies derived from patients in remission, uninvolved mucosa of colon adenocarcinoma, diverticular disease, as well as healthy individuals. The iNOS was heavily expressed on the apical surface of the epithelium at the top of the crypts with less expression in deeper epithelial cells of the crypts, while iNOS expression was not detectable in the lamina propria or in the inflammatory leukocytes closely associated with the epithelial cells. Similarly, Reynolds et al (Reynolds *et al.*, 1995), using in situ

hybridisation and immunohistochemistry have demonstrated high expression of iNOS localised to the surface epithelium and crypts in the mucosa from patients with ulcerative colitis. These results strongly suggest that the colonic epithelial cells are the major source of NO production and NOS activity that is increased in the mucosa from patients with ulcerative colitis (Middleton *et al.* 1993; Lundberg *et al.* 1994; Boughton-Smith *et al.* 1993) and this NO generation is in close association with, and probably contributory to, the inflammatory process. Our results with biopsies derived from newly diagnosed ulcerative colitis and infectious colitis patients extend and complement a parallel study by Singer et al. (1996) in which resected colon from patients with long standing intractable ulcerative colitis were examined. The similarity in these two studies is the heavy iNOS staining in the apical region of the superficial region of crypts in close association to areas of intense neutrophil infiltration of lamina propria and epithelium. In addition Singer et al. (1996) have noted iNOS expression in the deeper epithelial cells of the crypts as well as the occasional iNOS expression by the infiltrating mononuclear and polymorphonuclear cells within the lamina propria. Whether this increased pattern of iNOS distribution reflects the chronicity and untreatable nature of the ulcerative colitis in the Singer et al. study compared to our own remains to be determined. However, another complementary observation between the two studies is that we noted an absence of iNOS expression by the epithelial cells in the non-inflamed mucosa of patients with diverticular disease, while Singer et al. observed iNOS expression in inflamed mucosa of patients with diverticulitis and that iNOS expression was not present in colonic biopsies from patients with infectious colitis in total remission after treatment. Taking these results together it is clear that iNOS expression by colonic epithelial cells is only present when there is an underlying inflammatory component within the colonic mucosa.

In support of our immunohistochemical findings, we have demonstrated that human HT-29 colonic epithelial cells in response to specific combinations of cytokines express iNOS mRNA and produce large quantities of nitrite. The expression of iNOS activity in cells and tissues is thought to be controlled by a combination of cytokines and the profile of cytokine responsiveness varies with the cell type. In our study unstimulated HT-29 cells produced a small amount of nitrite in a time dependent manner, which appeared to be due to constitutive NOS as no iNOS mRNA expression was detected and the production of nitrite was unaffected by the protein synthesis inhibitor cycloheximide. None of cytokines added alone increased the nitrite generation. These results are similar with that found in cultured human hepatocytes (Nussler *et al.* 1992; Geller *et al.* 1993), lung epithelial cells (Asano *et al.* 1994; Robbins *et al.* 1994), and mesangial cells (Nicolson *et al.* 1993). In contrast, treatment of either human vascular smooth muscle cells (Junquero *et al.* 1992) with IL-1β, or human chondrocytes (Charles *et al.* 1993; Maier *et al.* 1994) with single proinflammatory cytokines IL-1β, IFN-γ, or TNF-α caused a marked increase in iNOS mRNA, iNOS protein, and nitrite generation. The combination of IL-1α and IFN-γ was the minimal stimulation required, while no other pair of cytokines was effective, for iNOS mRNA expression or significant increase of nitrite generation in HT-29 cells. Similar results were also obtained with human mesangial cells (Nicolson *et al.* 1993). Combinations of IL-1α and IFN-γ induced a concentration dependent nitrite generation and iNOS mRNA expression. Treatment of HT-29 cells with IL-1α/IFN-γ induced up to a 4 fold increase in nitrite generation, which is in contrast with human hepatocytes as the same combination of cytokines induced only 5% increase in nitrite generation (Nussler *et al.* 1992). The combination TNF-α/IL-1α/IFN-γ was most effective with nitrite levels increasing 12 fold compared to constitutive values.

The presence of these three signals produced the same high increase of nitrite production in human hepatocytes, lung epithelial cells, and mesangial cells. Studies of iNOS mRNA expression revealed that the TNF-α up-regulation of IL-1α/IFN-γ induced nitrite generation by HT-29 cells was at the post-transcriptional level. Cycloheximide, a protein synthesis inhibitor, has been reported to inhibit the nitrite generation in human cells treated with cytokines (Junquero *et al.* 1992; Nicolson *et al.* 1993; Palmer *et al.* 1993). Similarly in our study pretreatment with cycloheximide reduced the nitrite production to constitutive levels, regardless of maximal stimulation with IL-1α/IFN-γ, suggesting that the nitrite evoked by these cytokines is dependent on de novo protein synthesis, probably the iNOS enzyme and/or essential peptide co-factors.

We believe that the increased nitrite generation is due to iNOS enzyme activity because this production was induced by a combination of cytokines and blocked by cycloheximide, a protein synthesis inhibitor. However more importantly, we have only detected increased nitrite generation with those treatments which induce the expression of iNOS mRNA. Time course studies with the combination of IL-1α and IFN-γ demonstrated that the nitrite generation peaked at 72h, while peak ratio of nitrite production occurs between 24-48h and slows between 48-72h. The different ratio of nitrite generation could represent peak protein translation or co-factors generation at 24-48h, which is necessary for optimum enzyme activity. Furthermore Northern analysis demonstrated that iNOS mRNA peaks at 24h and is undetectable by 72h, indicating a loss of iNOS mRNA from 24h onwards available for protein synthesis. These results are consistent with the findings from human hepatocytes (Geller *et al.* 1993; Nussler *et al.* 1992) in which the iNOS mRNA peaked at 8h after stimulation and the

nitrite generation peaked at 48h. The fact that the maximum nitrite generation occurs more than 24h after the peak of mRNA indicates that iNOS enzyme is stable and active for long after its synthesis. The gap between maximum enzyme message expression and maximum nitrite generation might suggest the requirement of de novo synthesis of a co-factor, e.g. tetrahydrobiopterin (Stuehr & Griffith, 1992; Nussler *et al.* 1992) for maximal activity. These data suggest that human colonic epithelial cells must now be considered together with human hepatocytes, smooth muscle cells, chondrocytes, lung epithelial cells, and mesangial cells as an important source of nitric oxide production.

Since both T-cells and T-cell-derived cytokines including IFN-γ, IL-4, and IL-10 have been detected in the mucosa of patients with inflammatory bowel disease (Lichtman & Sartor, 1993; Sartor, 1994; Kolios & Nakos, 1995) we have utilised the human colonic epithelial cell line HT-29 as a model to explore the regulation by T-cell derived cytokines of epithelial iNOS expression and activity. We have demonstrated for first time that IL-4 and IL-13, but not IL-10 produces a concentration related and a marked inhibition of nitrite generation, iNOS protein expression and iNOS mRNA induced in a human colonic cell line by the optimal cytokine cocktail of IL-1α/IFN-γ/TNF-α. However, it is likely that IL-4 and IL-13 are operating via distinct receptors as differential effects of IL-4 and IL-13 were observed (Callard *et al.* 1996). Firstly low doses of IL-13, but not IL-4 produced an enhanced nitrite generation when the sub-optimal stimuli of IL-1α/IFN-γ combination was used, although higher concentrations of IL-4 and IL-13 both inhibited nitrite generation in this system. Secondly, this differential effect of IL-4 and IL-13 was observed at the level of iNOS mRNA, namely low concentrations of IL-13, but not IL-4, enhanced iNOS mRNA expression, while

concentrations above 3 ng/ml of IL-4 and IL-13 produced a concentration related and marked inhibition of iNOS mRNA transcription. It is clear by taking the iNOS mRNA, iNOS protein and nitrite generation data into account that the inhibitory effect of IL-13 and IL-4 observed at low concentrations of each of these cytokines is dependent upon the presence of TNF-α, acting as a post-transcriptional stimulator of iNOS expression and activity. In contrast, the higher concentrations of both IL-4 and IL-13 are potent inhibitors of iNOS mRNA induction, which is entirely independent of the presence of TNF-α.

Interleukin-13 has been reported to inhibit nitric oxide production by activated murine peritoneal macrophages (Doyle *et al.* 1994) and mouse bone marrow-derived macrophages (Doherty *et al.* 1993). Recently Saura et al. (1996), have published that IL-13 inhibits iNOS expression in human mesangial cells after stimulation with a "cocktail" of pro-inflammatory cytokines and LPS. However, the mechanism responsible for this effect is not known. In our study IL-13 was found to have a dual effect on pro-inflammatory-derived iNOS expression and activity in HT-29 cells. As we discussed above, it inhibits, at all concentrations examined, the postranscriptional effect of TNF-α on IL-1α/IFN-γ-induced nitrite generation and in concentrations higher than 3 ng/ml it inhibits the IL-1α/IFN-γ-induced iNOS expression and activity, while at lower doses enhances the effect of IL-1α/IFN-γ on iNOS expression and activity by HT-29 cells. Similar up-regulating effect was observed for IL-4, which induces an increased secretion of nitrite by IFN-γ-activated human monocytes (Kolb *et al.* 1994). IL-4 has been reported to inhibit NO synthesis in mouse activated macrophages (Oswald *et al.* 1992), in murine macrophages (Cenci *et al.* 1993; Liew *et al.* 1991), and human mesangial cells (Saura *et al.* 1996). IL-4 shares many properties with IL-13 with regard to biological

activities and signal transduction pathways and in most cell types in which an IL-4 effect was seen, subsequent analysis has shown that IL-13 is also effective (Zurawski & De Vries, 1994). However these two cytokines have also different biological functions on the same cell lines. It has been reported that the binding sites for IL-4 and IL-13 on target cells are slightly different, but at least one binding protein is shared by both IL-4 and IL-13 receptors. Some cells, however, may express all three subunits for IL-4 and IL-13 receptors, while other cells may express different subunits or not all of them (Feng *et al.* 1995).

Interleukin-10 is considered as an anti-inflammatory cytokine, which was originally described as Th2-cell-derived cytokine. Recent studies have shown that IL-10 is produced by a variety of cells, including Th2 cells, Ly-1 B cells, mast cells and cells of the macrophage lineage (Moore *et al.* 1993). This cytokine at concentrations of 10 ng/ml or less is an effective inhibitor of cytokine generation by IFN-γ and/or LPS stimulated human monocytes (De Waal Malefyt *et al.* 1991), prostaglandin E_2 production by IL-1 or LPS activated human monocytes (Poole *et al.* 1995) and chemokine generation by polymorphonuclear leukocytes treated with LPS (Kasama *et al.* 1994). IL-10 has been found to inhibit NO production by mouse activated macrophages (Oswald *et al.* 1992; Cenci *et al.* 1993). However, we found that IL-10 throughout the concentration range of 0.1 to 10 ng/ml had no effect on pro-inflammatory cytokine-induced iNOS expression and activity by the colonic epithelial cell line HT-29. Despite the absence of any effect of IL-10 on our cell model, our findings give a strong evidence that the T-cell derived cytokines modulate the pro-inflammatory cytokines derived iNOS expression and activity in colonic epithelial cells and might be involved in intestinal inflammation.

Although our results were obtained with a colonic epithelial cell line, which has many characteristics in common with "normal" colonic epithelial cells (Chantret *et al.* 1988) the possibility exists that "normal" cells may behave differently to tumour cells. Thus, colonic biopsies were cultured under similar conditions to explore the ability of normal colonic mucosa to produce nitrite and to examine the agents, which regulate this generation. We have demonstrated for first time that un-stimulated cultures of colonic biopsies from normal bowel mucosa produced a constitutive amount of nitrite. Stimulation with pro-inflammatory cytokines induced a 3fold increase of this production. This increase of nitrite generation probably might be due to induction of iNOS in colonic epithelial cells , as the increase NO production in IBD (Middleton *et al.* 1993; Lundberg *et al.* 1994) is in close association with a strong iNOS activity mainly in colonic epithelium (Boughton-Smith *et al.* 1993; Singer *et al.* 1996; Kolios *et al.* 1997). The addition of anti-inflammatory cytokine IL-13 potently inhibited the nitrite generation by colonic mucosa in a similar manner to nitrite generation by colonic epithelial cells HT-29. Similarly, interleukin-13 has been reported to inhibit nitric oxide production by activated murine peritoneal macrophages (Doyle *et al.* 1994) and mouse bone marrow-derived macrophages (Doherty *et al.* 1993). Saura et al. (1996), have published that IL-13 inhibits iNOS expression in human mesangial cells after stimulation with a "cocktail" of pro-inflammatory cytokines and LPS.

In conclusion, we have demonstrated in this study that colonic epithelial cells HT-29 express iNOS and produce nitrite after induction with cytokines, while T-cell derived cytokines down-regulate iNOS expression and activity. Furthermore, our immunohistochemical study has shown that inflamed colonic mucosa from patients with intestinal inflammation expressed iNOS activity and colonic epithelial cells were the main source of this expression. In marked

contrast, uninflamed epithelium from controls did not express iNOS activity. These data taken together with our results from cultures of colonic biopsies from normal bowel mucosa indicate the involvement of colonic epithelium in NO production and suggest a crucial role of pro-inflammatory and T-cell derived cytokines in iNOS expression and activity in colonic mucosa. The stimulation of colonic epithelial cells by inflammatory mediators seem to trigger the increased production of NO in colonic mucosa in intestinal inflammation, while the T-cell derived cytokines may have a regulatory effect on NO generation (Fig. 18).

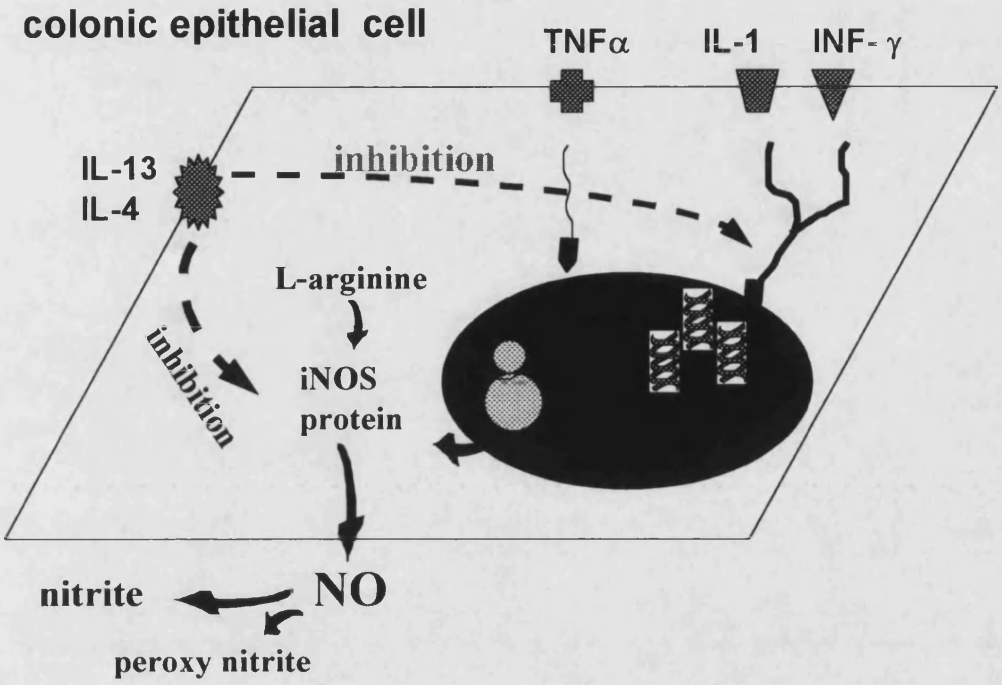

Figure 18 Nitric oxide production by colonic epithelial cells after stimulation with pro-inflammatory cytokines. Inhibitory effect by the T cell derived cytokines IL-4 and IL-13.

3.2 CHEMOKINE EXPRESSION BY COLONIC EPITHELIAL CELLS

3.2.1 Chemokine secretion from HT-29 cell line in response to cytokines

Confluent monolayers of HT-29 cells were treated with either IL-1α (0.1-30 ng/ml) or TNF-α (1-100 ng/ml) or IFN-γ (10-300 U/ml) or IL-13 (0.1-10 ng/ml) or IL-4 (0.1-10 ng/ml) or IL-10 (0.1-10 ng/ml) for 24 hours. Supernatants were collected and IL-8, MCP-1 and RANTES secretion was measured by ELISA. Un-stimulated cells did not produce detectable levels of chemokines (below 200 pg/ml).

The generation of IL-8 was found to be markedly up-regulated in a concentration dependent manner by either the IL-1α or TNF-α (Fig. 19a, 19b). Interestingly, maximal concentration of TNF-α produced twice as much IL-8 (164.4 ± 6.9 ng/ml) as was induced by maximal concentration of IL-1α (79.6 ± 8.4 ng/ml). Furthermore, neither IFN-γ nor IL-13 nor IL-4 nor IL-10 stimulated IL-8 generation, when added alone to HT-29 cells. Finally, both IL-1α (30 ng/ml) and TNF-α (100 ng/ml) induced a time dependent generation of IL-8 with significant amounts detected by 4 hours and peak production with each cytokine was at 24 hours (Fig. 20).

Pro-inflammatory cytokines and T cell derived cytokines added alone did not induce MCP-1 or RANTES secretion by HT-29 cells. The combination of TNF-α/IFN-γ was the minimal stimulation for MCP-1 an RANTES generation while no other pair of cytokines was effective. This combination produced a concentration dependent MCP-1 and RANTES secretion with a

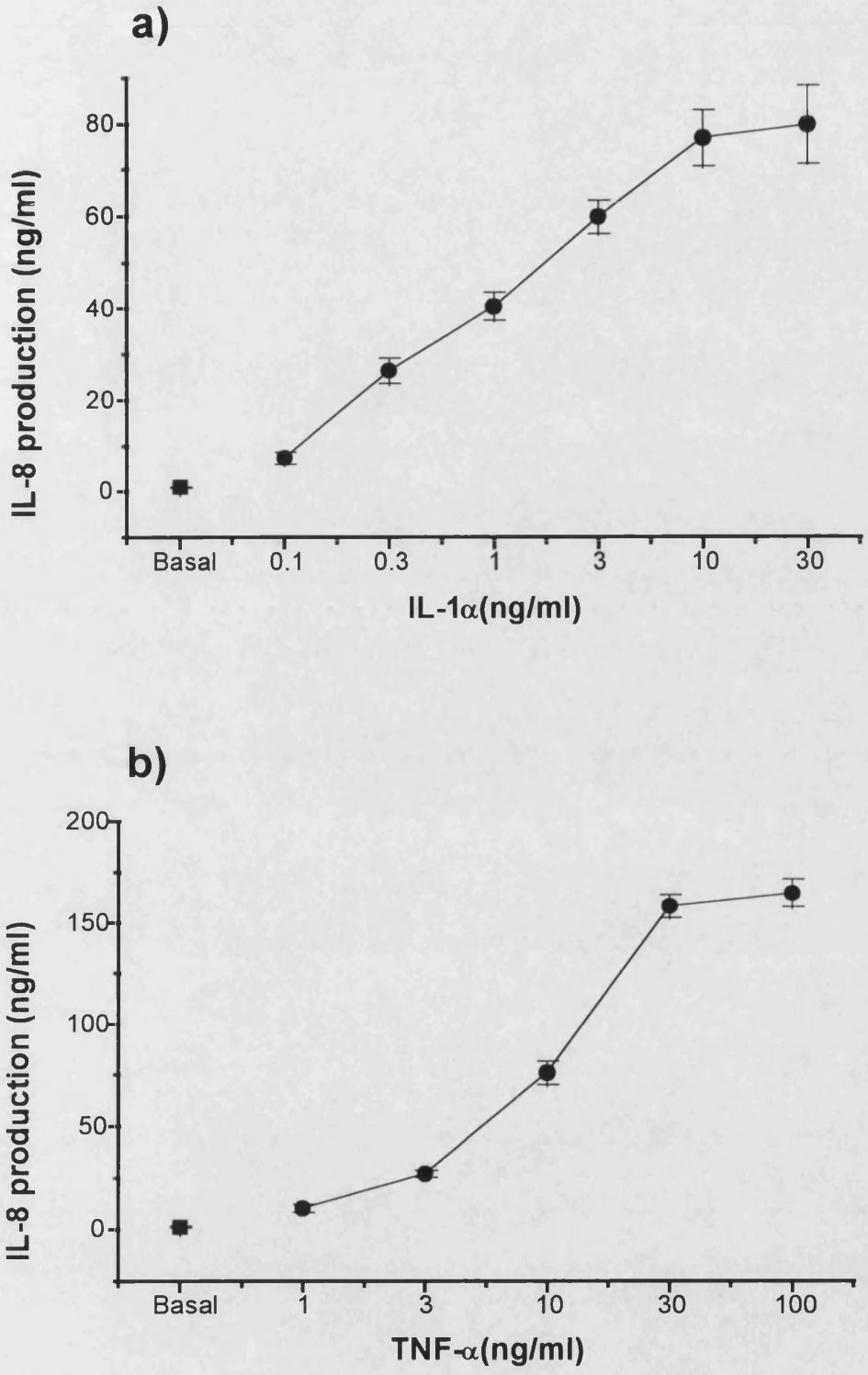

Figure 19 Production of IL-8 by HT-29 cells following stimulation for 24h with IL-1α (a), or TNF-α (b), or vehicle (basal). Each point is the mean ± SEM of three experiments.

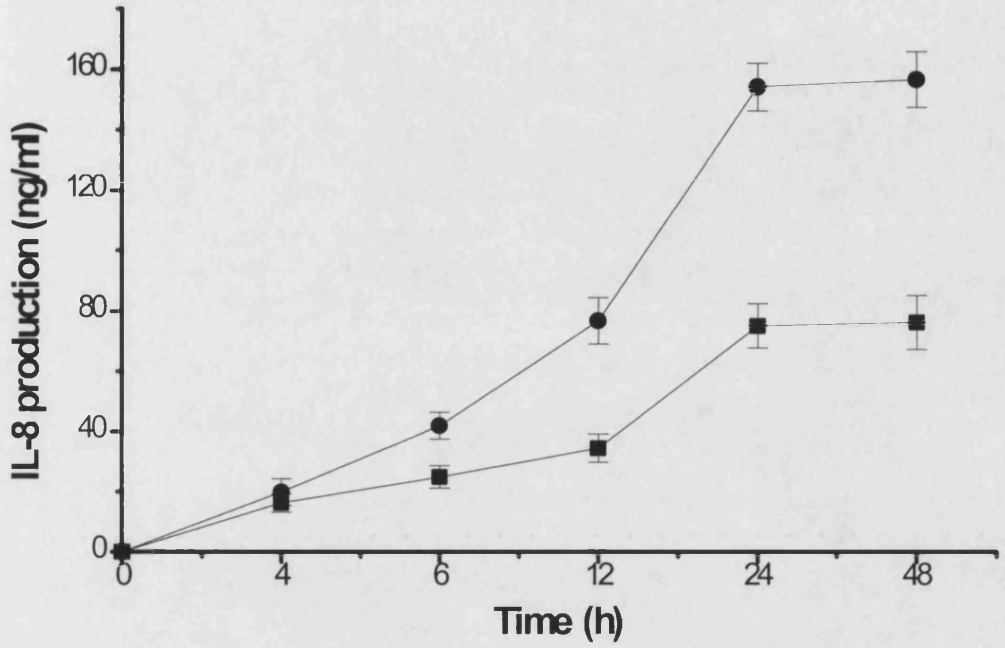

Figure 20 The time course of IL-8 production by HT-29 cells following stimulation with IL-1α (■, 30 ng/ml), or TNF-α (●, 100 ng/ml),. Each point is the mean ± SEM of three experiments.

peak production of 8.09 ± 0.55 ng/ml and 19.63 ± 1.4 ng/ml, respectively, after a maximal stimulation with TNF-α (100ng/ml) / IFN-γ (300U/ml) at 48 h. The same stimulation induced a stronger IL-8 mRNA expression and higher levels of IL-8 secretion (e.g. 283 ± 18 ng/ml of IL-8) (Fig. 21).

3.2.2 Chemokine mRNA expression by HT-29 cells

Chemokine mRNA expression in epithelial cells was examined. In un-stimulated cells no chemokine transcripts were detected. IL-1α induced IL-8 mRNA expression peaked at 1h and declined rapidly to a very low expression by 24h (Fig. 22). In contrast, TNF-α-induced IL-8 mRNA peaked at the same time, but remained high for at least 12h (Fig. 23). No single cytokine was capable of inducing MCP-1 or RANTES mRNA expression. The combination of TNF-α/IFN-γ was the minimal stimulation for MCP-1 and RANTES mRNA expression, while no other pair of cytokines was effective. The same stimuli induced a strong IL-8 mRNA expression. Characteristically HT-29 cells showed a delayed MCP-1 mRNA expression, which started at 6 h and peaked at 12 h and a later on RANTES mRNA expression, which started at 12 h and peaked at 24 h in comparison with the early IL-8 mRNA at 1 h (Fig 24).

3.2.3 Synergistic effect of IL-13 and IL-1α on IL-8 secretion

Possible modulatory effects of IL-10 and IL-13 on IL-1α or TNF-α-induced IL-8 production were examined. Confluent monolayers of HT-29 cells, after 1 hour pre-treatment with either IL-13 (0.1-10 ng/ml) or IL-10 (0.1-10 ng/ml), were treated with either IL-1α or TNF-α. Cells

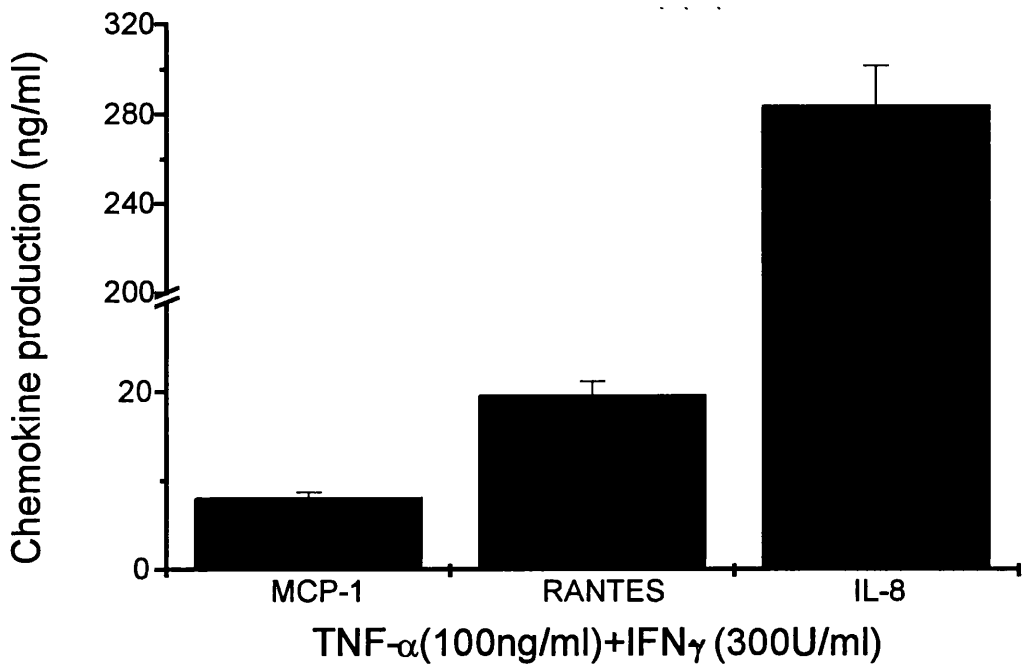

Figure 21 Chemokine secretion by HT-29 cells after 48 hours incubation at 37° C in the presence of the proinflammatory cytokines TNF-α and IFN-γ. Each bar is the mean ± SEM of three experiments.

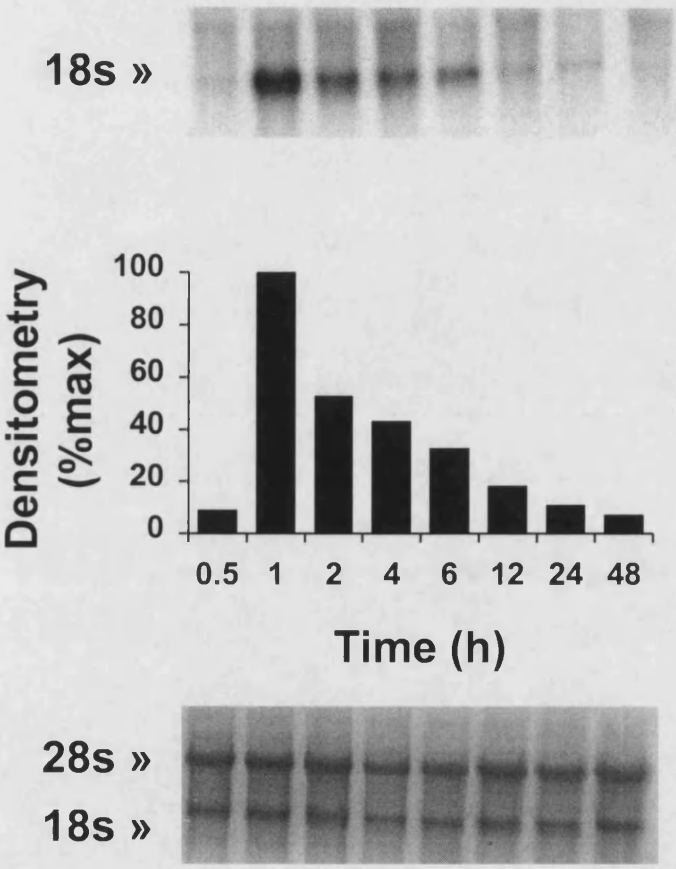

Figure 22 Northern blot analysis of time course of IL-8 mRNA expression in HT-29 cells stimulated with IL-1α (3 ng/ml). The top panel of each figure is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

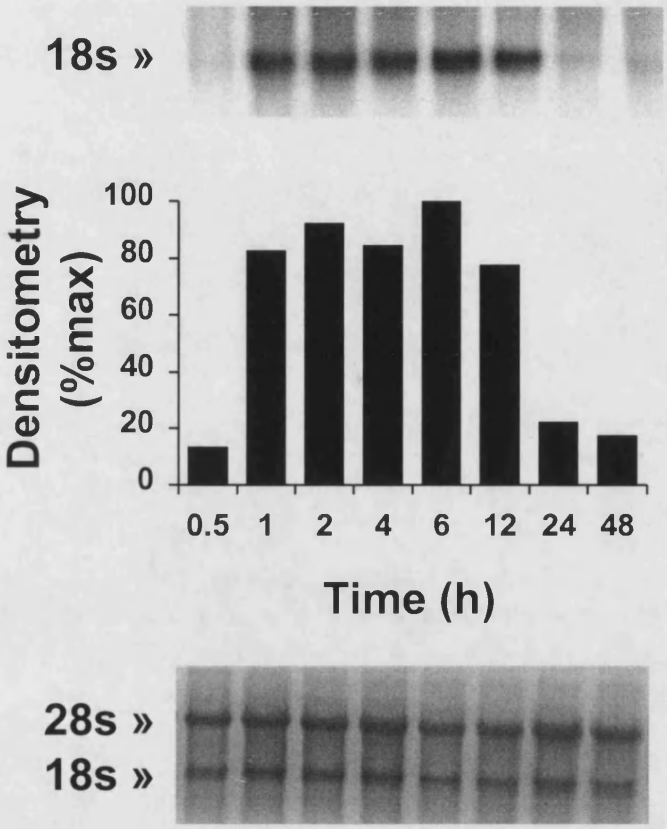

Figure 23 Northern blot analysis of time course of IL-8 mRNA expression in HT-29 cells stimulated with TNF-α (30 ng/ml). The top panel of each figure is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

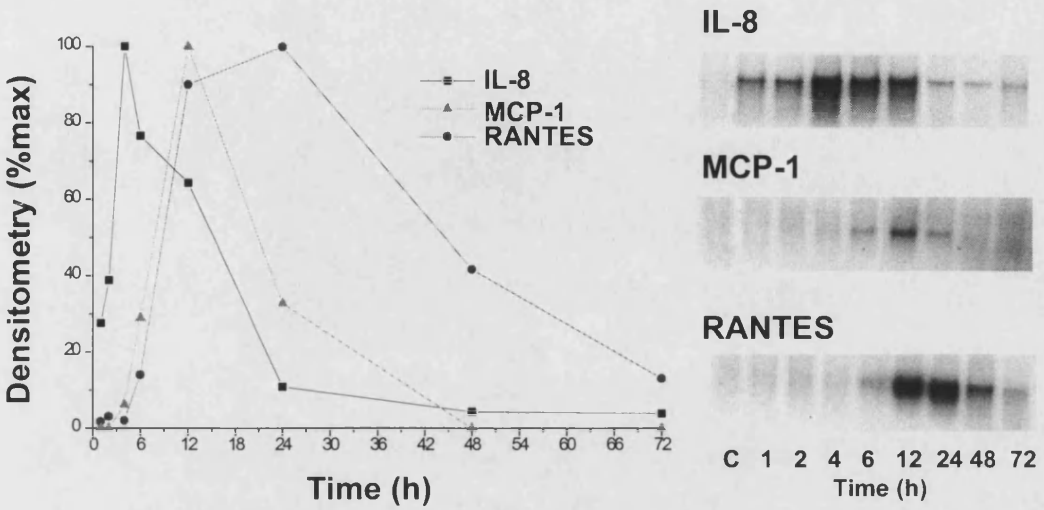

Figure 24 Northern blot analysis of time course of chemokine mRNA expression in HT-29 cells stimulated up to 72 hours with the combination IFN-γ (300U/ml) + TNF-α (100 ng/ml). Representative of three experiments.

were incubated for 24 hours and then IL-8 secretion was measured in supernatants by ELISA. At all concentrations of IL-1α examined, IL-13 (1-10ng/ml) produced a significant enhancement of IL-8 generation ($p<0.05$ -$p<0.01$) (Fig. 25), for example the presence of IL-13 (10ng/ml) induced approximately twice as much IL-8 (91.5 ± 3.5 ng/ml) compared to that produced by IL-1α (3 ng/ml) alone (57.1 ± 1.8 ng/ml, $p<0.01$) (Fig. 19a). In marked contrast the same concentration range of IL-13 had no effect on TNF-α induced IL-8 generation (Fig. 26). Furthermore, IL-10 (0.1-10 ng/ml, n=3) was found to have no effect on either IL-1α or TNF-α induced IL-8 production (data not shown). Synergistic effect of IL-4 and IL-1α on IL-8 secretion by HT-29 cells was subsequently examined by Dr Adrian Minty, Sanofi Recherche, France, and it was found similar to the effect of IL-13 (personal communication).

To determine whether the synergistic effect of IL-13 with IL-1α on IL-8 generation was at the mRNA level, cells were pre-treated for 1h with IL-13 (5 ng/ ml), then IL-1α (3 ng/ml) was added and mRNA expression was measured from 1 to 12h post IL-1α addition. IL-13 was found to prolong the IL-1α-induced mRNA expression in HT-29 cells (Fig. 22, 27), while it was without effect on TNF-α-induced IL-8 mRNA expression (Fig. 23, 28).

Experiments using the protein synthesis inhibitor cycloheximide were then conducted to determine whether the IL-13-induced prolongation of IL-8 mRNA was due to de novo protein synthesis. HT-29 monolayers were treated with cycloheximide (5 mg/ml) for 2 hours and then IL-1α (3 ng/ml) was added alone or after 1 hour pre-treatment with IL-13 (5 ng/ml), then total RNA was extracted at 1, 2, 4, 6 and 12h post IL-1α addition. The addition of cycloheximide was without effect on the IL-13-induced prolongation of IL-1α stimulated IL-8 mRNA

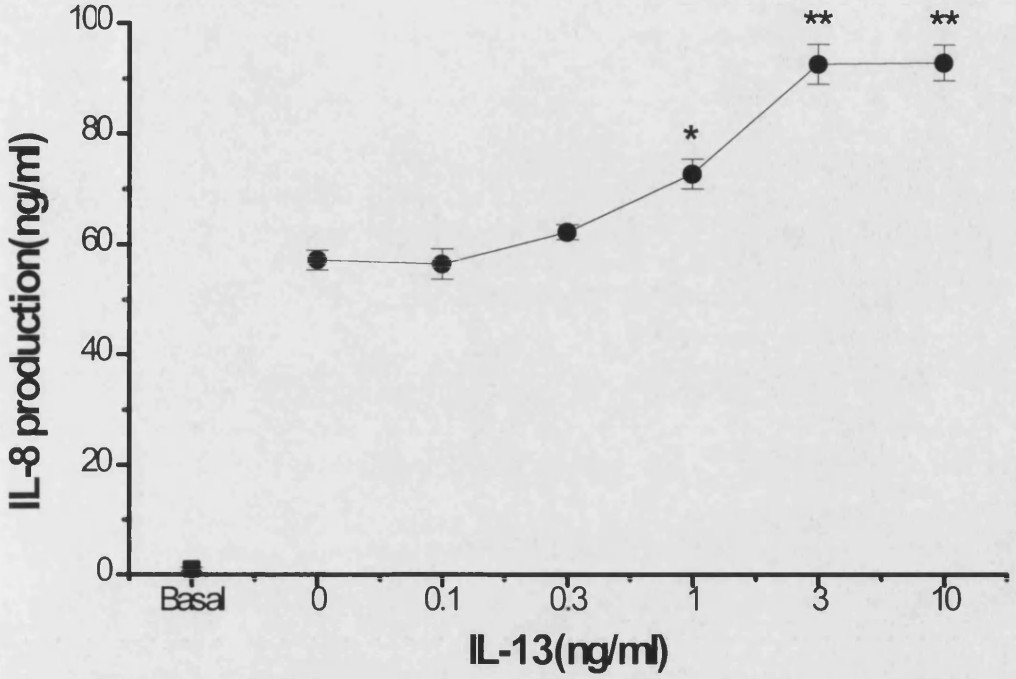

Figure 25 Effect of IL-13 (0.1-10 ng/ml) on IL-1α (●, 3 ng/ml) induced IL-8 generation by HT-29 cells after 24h treatment. Each point is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$).

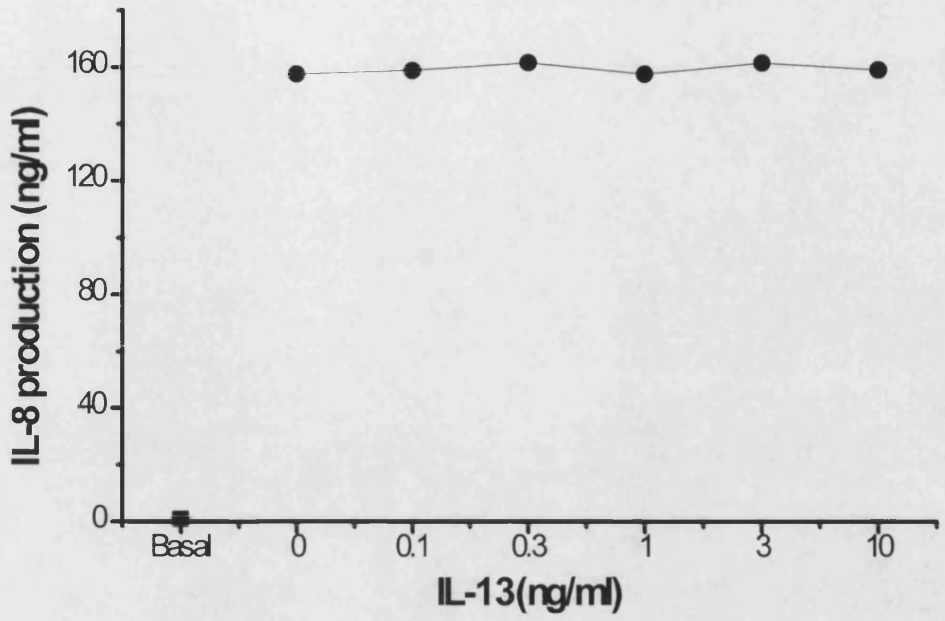

Figure 26 Effect of IL-13 (0.1-10 ng/ml) on TNF-α (●, 30 ng/ml) induced IL-8 generation by HT-29 cells after 24h treatment. Each point is the mean ± SEM of three experiments (* $p<0.05$, ** $p<0.01$).

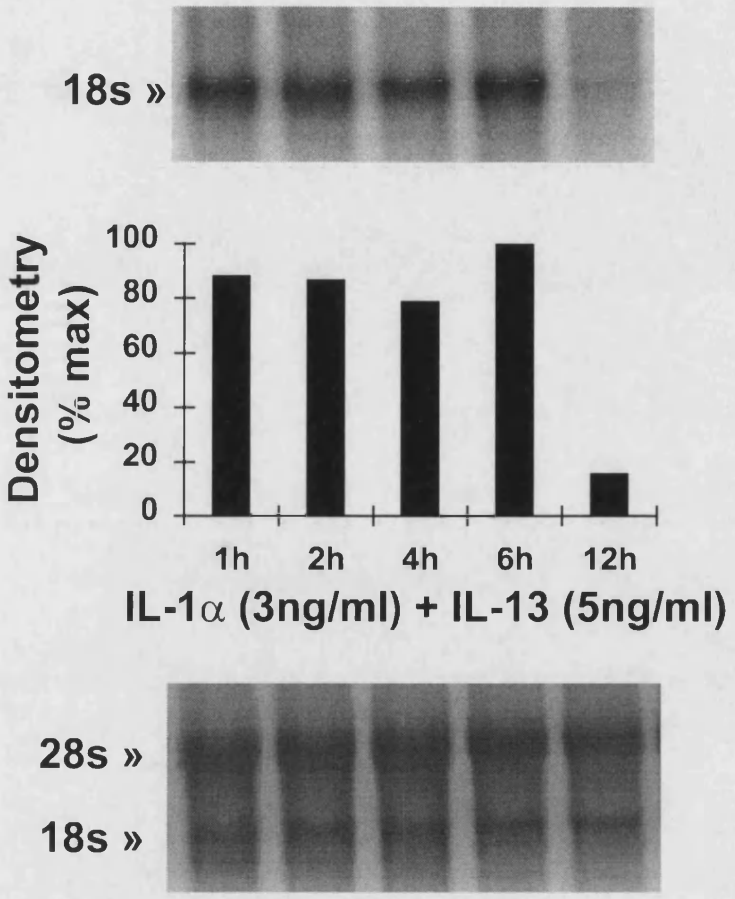

Figure 27 Effect of IL-13 on IL-1α induced IL-8 mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 (5 ng/ml) and then IL-1α (3 ng/ml) was added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

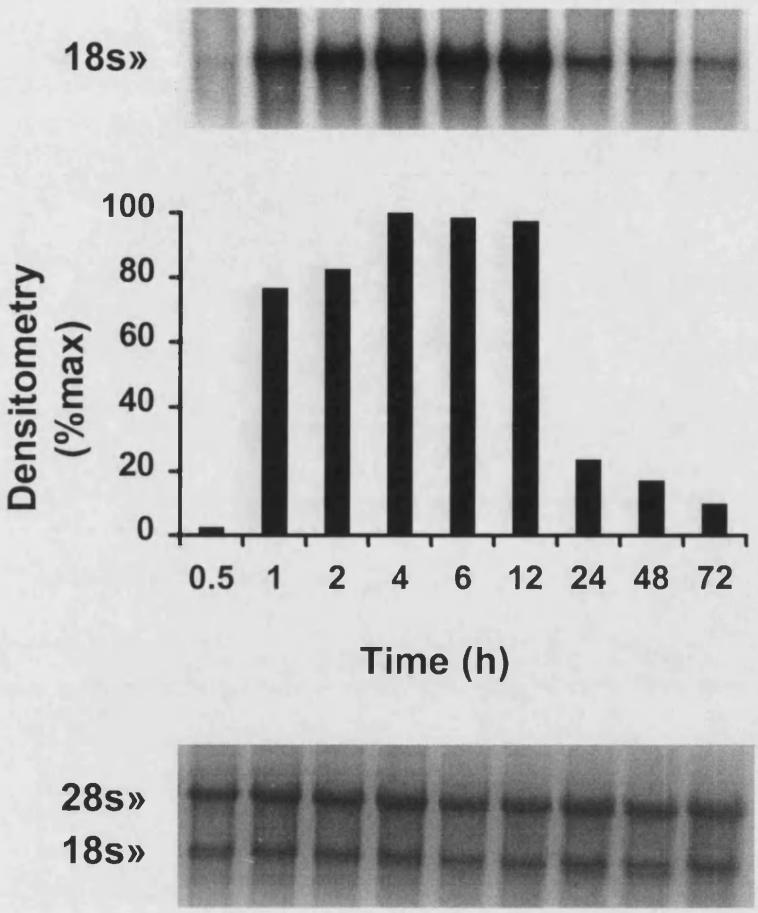

Figure 28 Effect of IL-13 on TNF-α induced IL-8 mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 (5 ng/ml) and then TNF-α (30 ng/ml) was added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

expression (compare Fig. 27 and Fig. 29). Although a small prolongation of IL-1α-induced IL-8 mRNA was produced by cycloheximide (compare Fig. 22 and Fig. 30), this prolongation was much weaker compared to that induced by IL-13 (Fig. 27).

The above findings demonstrate that IL-13 potentiates the IL-1α-derived IL-8 expression in colonic epithelial cells. The expression of most cytokine mRNA is regulated at the transcriptional or at the mRNA stability level. To determine the mechanism of IL-13-induced regulation, we examined the effect of IL-13 on the $t_{1/2}$ of IL-8 mRNA derived from IL-1α stimulated HT-29. After 1 hour incubation with IL-1α (3 ng/ml) in the presence (1h pre-treatment) of IL-13 (5 ng/ml) or vehicle, actinomycin-D (5 mg/ml) was added to the cultures to block further transcription and total RNA was extracted at specific time points. The expression of ß-actin mRNA is constitutive, thus its expression is not blocked by actinomycin-D. Analysis of the time course of the IL-8 mRNA / β-actin mRNA ratios in the presence of actinomycin-D provides a measure of the stability ($t_{1/2}$) of IL-1α-induced IL-8 mRNA independent of transcription. Comparison of the IL-8 mRNA / β-actin mRNA ratio in the presence or absence of IL-13 followed by the addition of actinomycin-D demonstrated that IL-13 did not affect IL-8 mRNA $t_{1/2}$ (Fig. 31). Therefore the site of increased IL-1α-induced IL-8 mRNA expression in the presence of IL-13 (Fig. 27) is at the level of transcription.

3.2.4 Differential effect of T cell derived cytokines on C-X-C and C-C chemokines by HT-29 cells

To examine the modulatory effect of T-cell derived cytokines on chemokine expression by

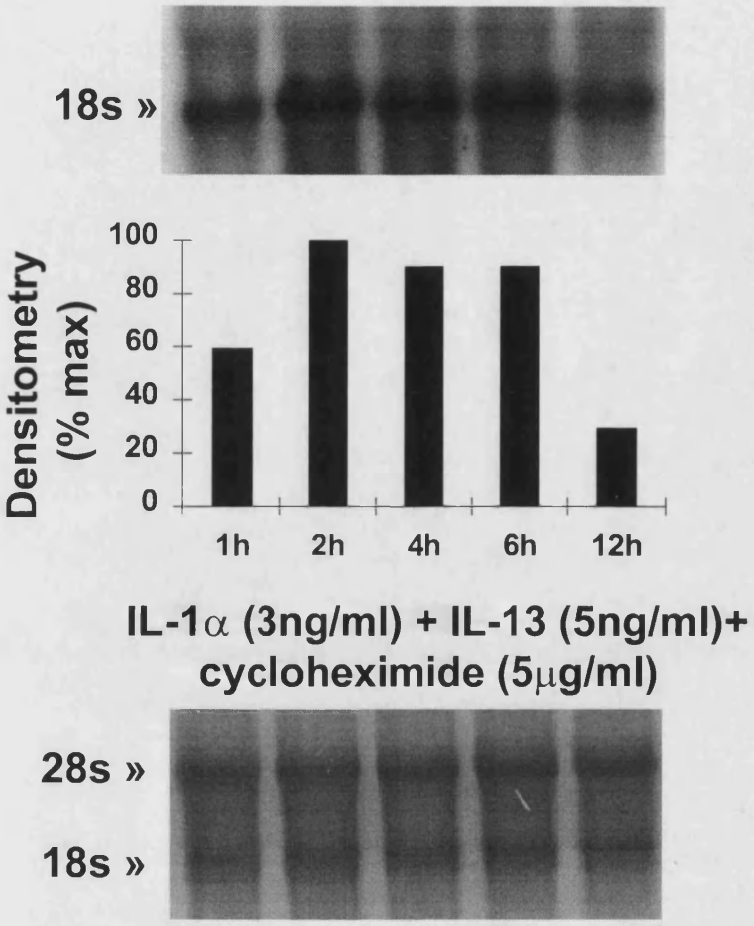

Figure 29 Effect of IL-13 on IL-1α induced IL-8 mRNA expression in HT-29 cells in the presence of cycloheximide. After 1 hour pretreatment with cycloheximide (5 μg/ml), IL-13 (5 ng/ml) was added and the incubation continued for 1 hour. Then IL-1α (3 ng/ml) was added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

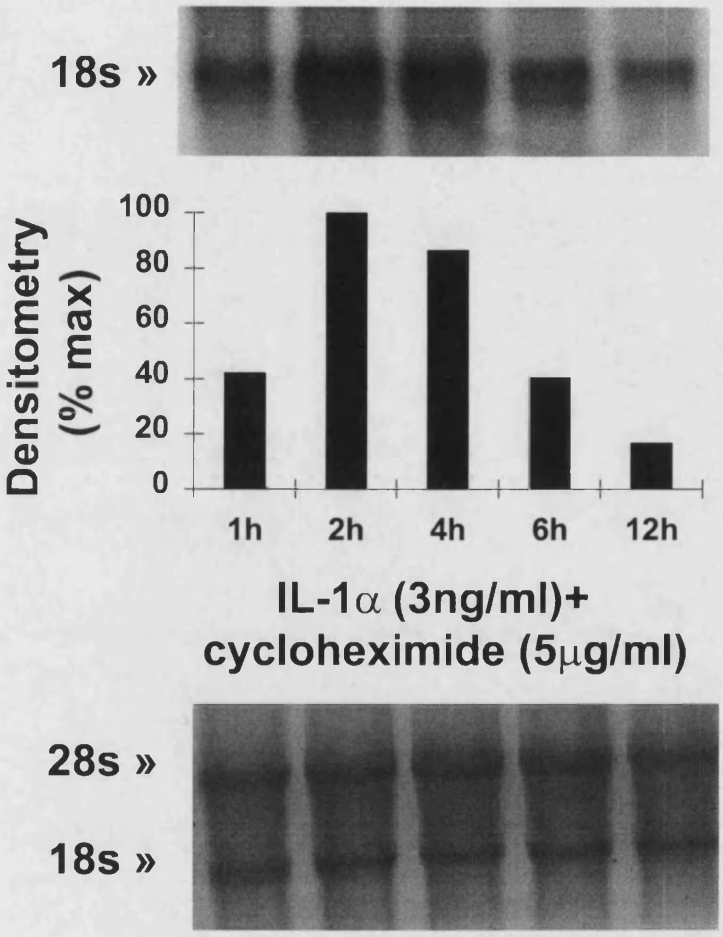

Figure 30 Effect of cycloheximide (5 μg/ml) on IL-1α induced IL-8 mRNA expression in HT-29 cells. Cells were pretreated for 2 hours with cycloheximide (5 μg/ml) and then IL-1α (3 ng/ml) was added. Total RNA extracted at 1, 2, 4, 6 and 12 h and IL-8 mRNA was analyzed by Northern blotting. Representative of three experiments.

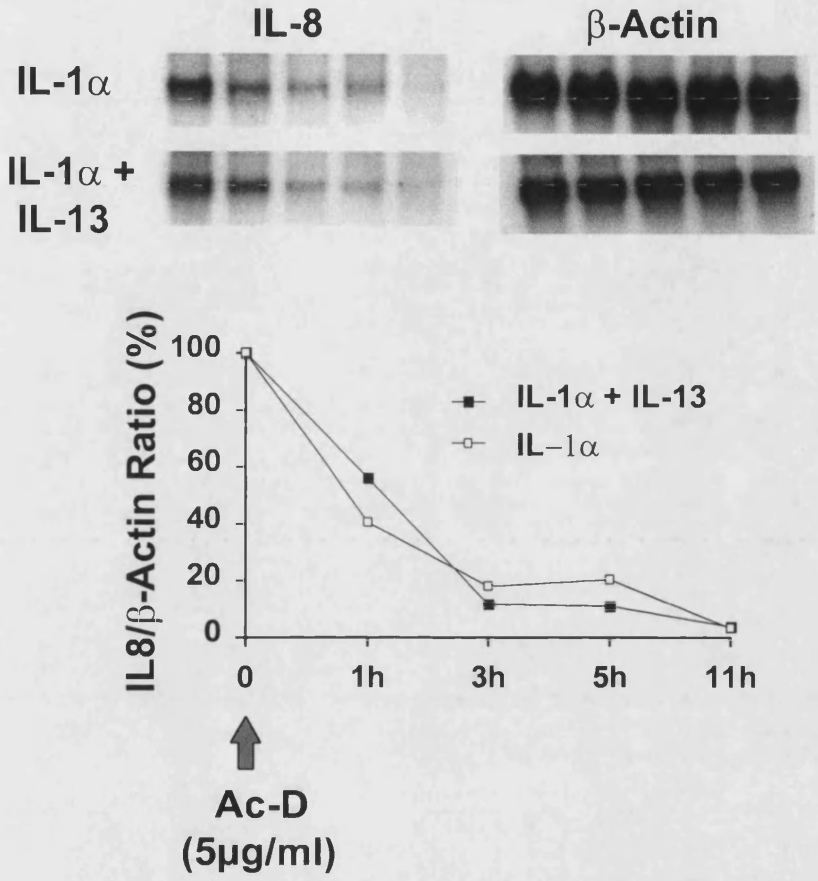

Figure 31 The $t_{1/2}$ of IL-8 mRNA determined at 1 hour after stimulation with IL-1α. Cells were stimulated for 1 hour with IL-1α (3 ng/ml) in the presence or absence of IL-13 (5 ng/ml). Then, actinomycin-D (5 μg/ml) was added and the decay of IL-8 mRNA was assessed by Northern blot analysis. The $t_{1/2}$ of IL-1α-induced IL-8 mRNA in the presence or absence of IL-13 were 1.05 h and 0.9 h, respectively. Representative of two experiments.

HT-29 cells growth arrested cultures were treated with proinflammatory cytokines added after 1h pre-treatment with various concentrations (0-30 ng/ml) of IL-13, IL-4 or IL-10. Pre-treatment with IL-13 reduced the TNF-α/IFN-γ-induced C-C chemokine mRNA expression and secretion. The presence of IL-13 reduced the MCP-1 production from 8.09 ± 0.55 ng/ml to 3.40 ± 0.39 ng/ml (Fig. 32) and the RANTES production from 19.63 ng/ml ± 1.44 ng/ml to 9.89 ± 0.59 ng/ml (Fig. 33). In marked contrast IL-13 had no effect on TNF-α/IFN-γ-induced IL-8 production by HT-29 cells induced by the same stimuli (Fig. 34). Examining the effect of IL-13 in the TNF-α/IFN-γ-induced chemokine mRNA expression we found similar inhibitory effect of IL-13 on C-C chemokine expression (Fig. 35,36), but not on TNF-α/IFN-γ-induced IL-8 expression by HT-29 cells (Fig. 37). IL-4 was found to share the same properties with IL-13 on chemokine mRNA expression and production by the HT-29 colonic epithelial cells (Fig. 38,39). Finally IL-10 was no effective on either C-C or C-X-C chemokine expression and secretion (Fig. 40).

3.2.5 Immunohistochemical study of colonic biopsies

Colonic biopsies of normal, non inflamed mucosa (8 out of 8 cases), derived from patients with diverticular disease, colon adenocarcinoma and healthy individuals were found negative for IL-8 expression (Fig. 41A). In all cases (n=15) of intestinal inflammation (ulcerative colitis, n=12 and infectious colitis, n=3) the epithelial cells markedly expressed IL-8, while a number of positive cells were found in lamina propria (Fig. 41B, 41C). Characteristically the IL-8 expression was located in the basal part of epithelial cells and it was in close association with the neutrophil infiltration of lamina propria and epithelium (Fig. 41B). Interestingly, biopsies

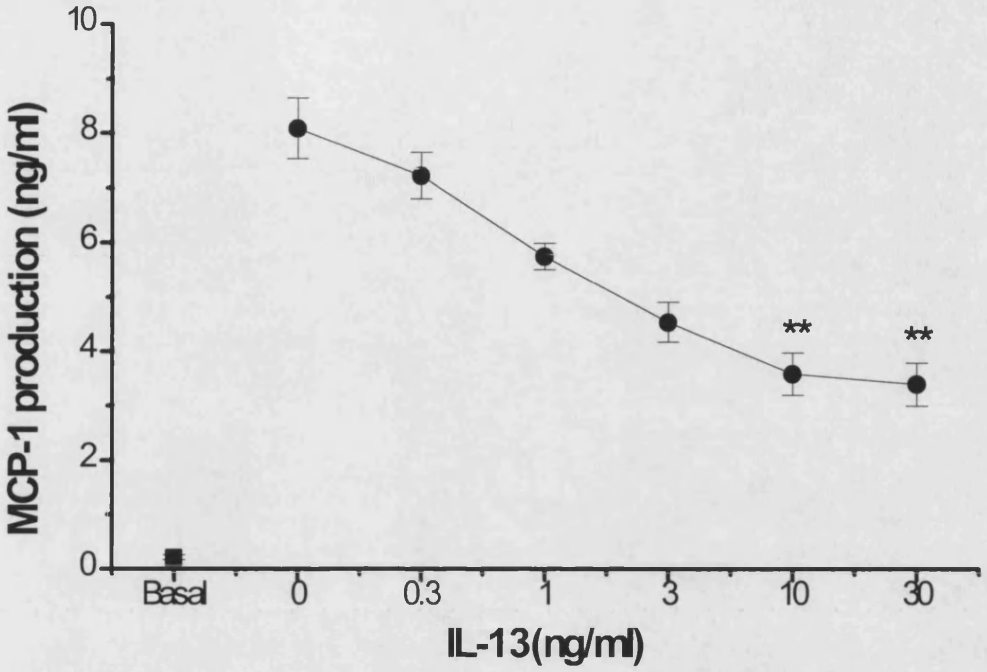

Figure 32 The effect of increasing concentrations of IL-13 (0.1-30 ng/ml) on IFN-γ(300U/ml)/TNF-α(100ng/ml)-induced MCP-1 generation by HT-29 cells after 24 h treatment. Each point is the mean ± SEM of three experiments (** $p<0.01$).

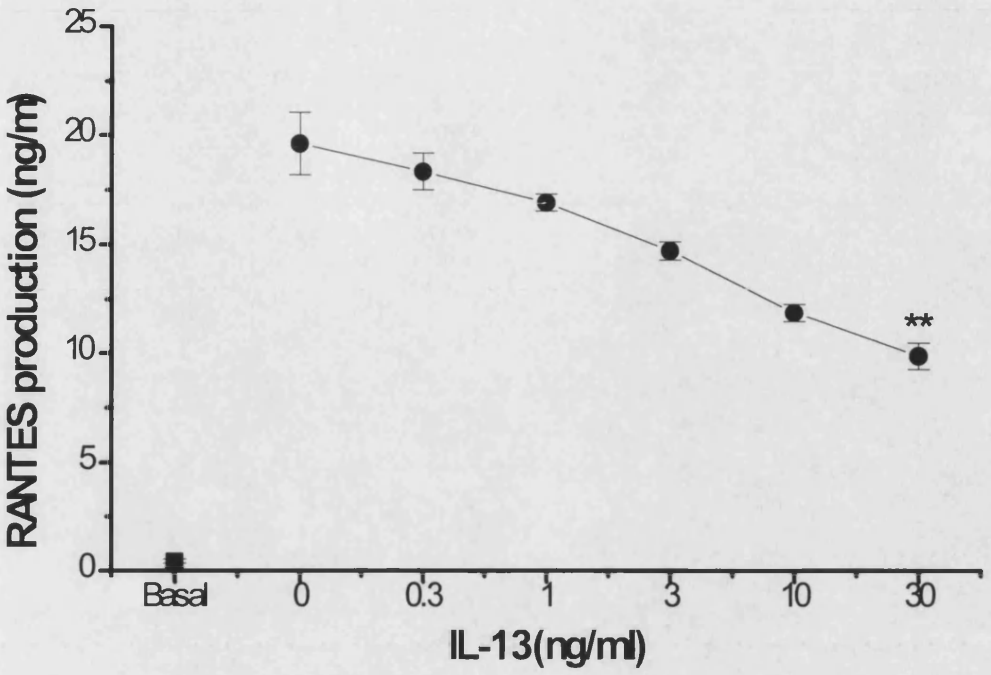

Figure 33 The effect of increasing concentrations of IL-13 (0.1-30 ng/ml) on IFN-γ(300U/ml)/TNF-α(100ng/ml)-induced RANTES generation by HT-29 cells after 24 h treatment. Each point is the mean ± SEM of three experiments (** $p<0.01$).

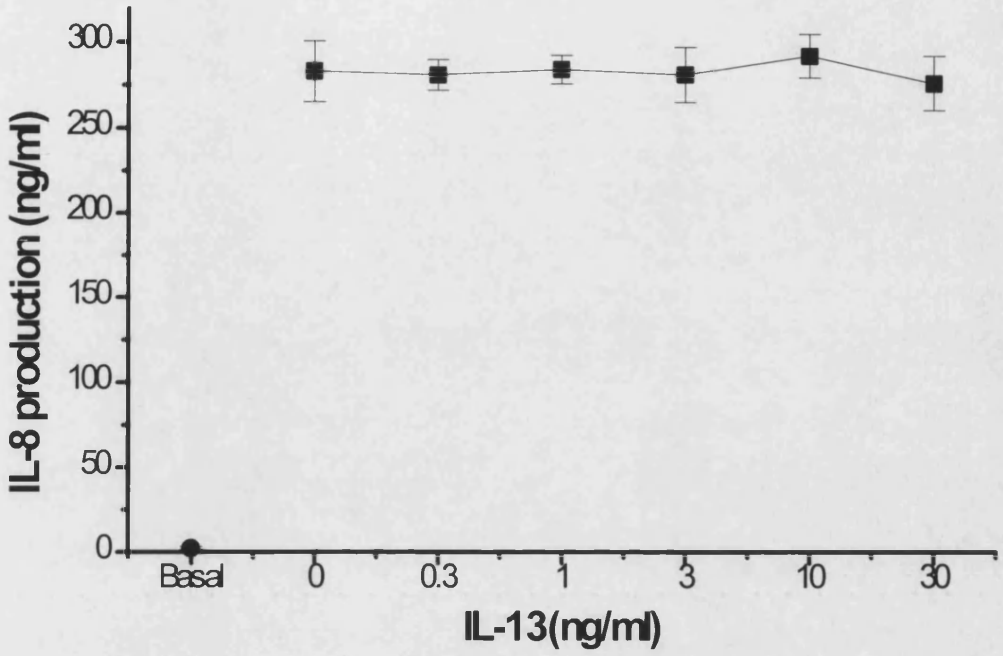

Figure 34 The effect of increasing concentrations of IL-13 (0.1-30 ng/ml) on IFN-γ(300U/ml)/TNF-α(100ng/ml)-induced IL-8 generation by HT-29 cells after 24 h treatment. Each point is the mean ± SEM of three experiments.

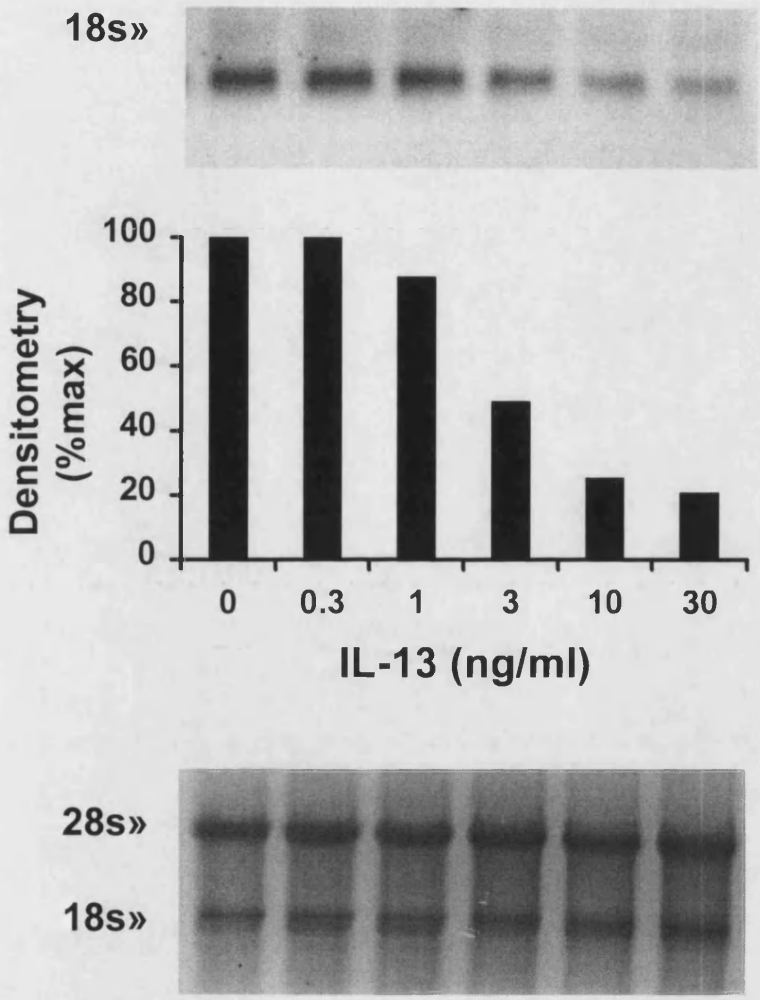

Figure 35 Effect of increasing concentrations of IL-13 on TNF-α/IFN-γ induced MCP-1 mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 and then TNF-α(100ng/ml)/IFN-γ(300 U/ml) were added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

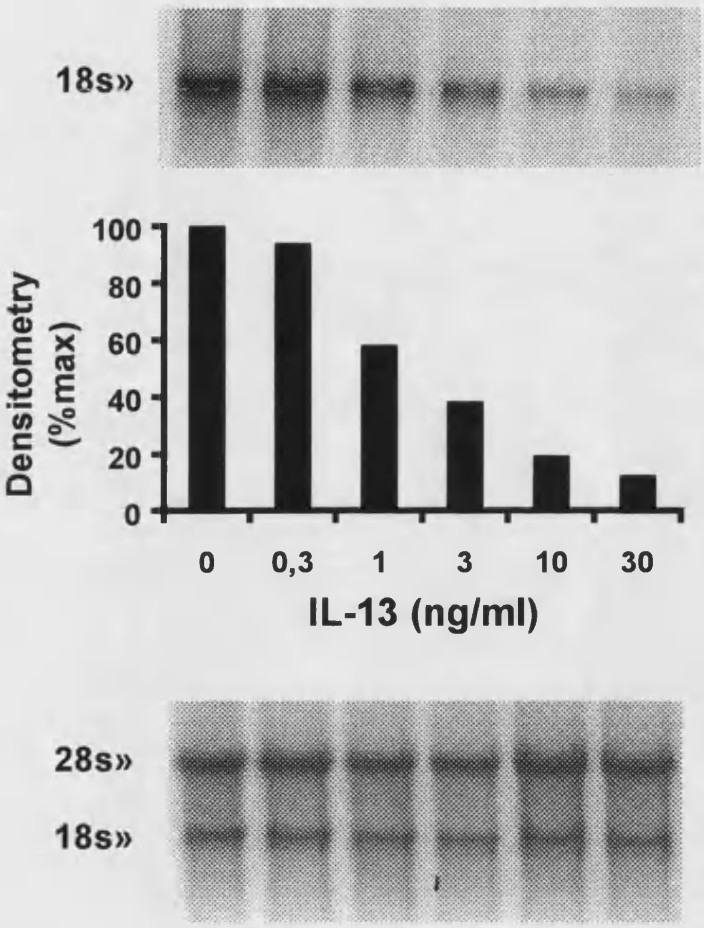

Figure 36 Effect of increasing concentrations of IL-13 on TNF-α/IFN-γ induced RANTES mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 and then TNF-α (100ng/ml)/IFN-γ (300 U/ml) were added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

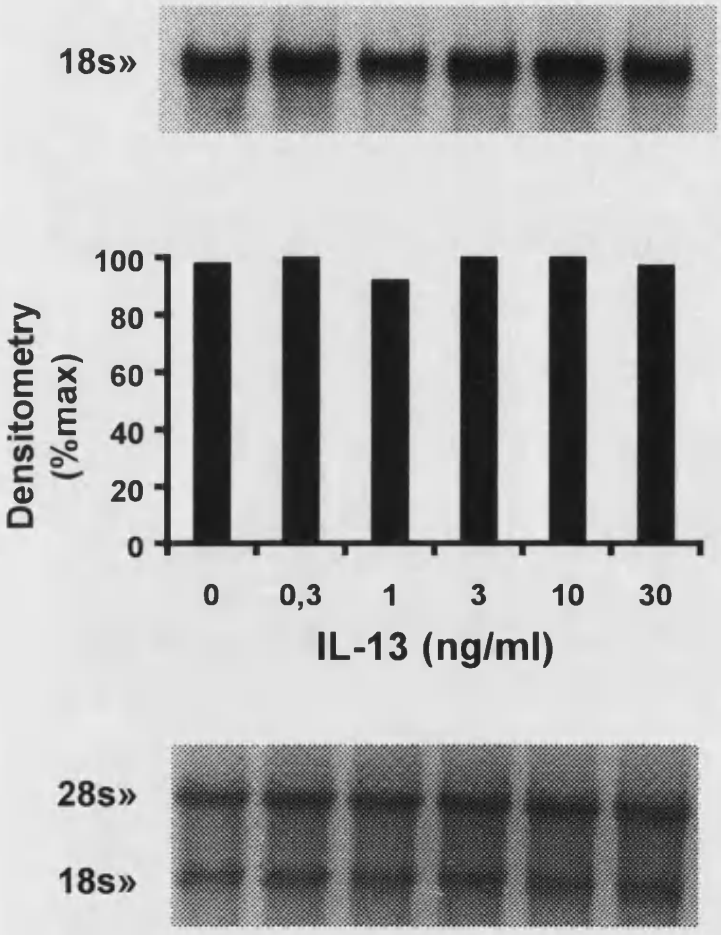

Figure 37 Effect of increasing concentrations of IL-13 on TNF-α/IFN-γ induced IL-8 mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 and then TNF-α (100ng/ml)/IFN-γ (300 U/ml) were added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

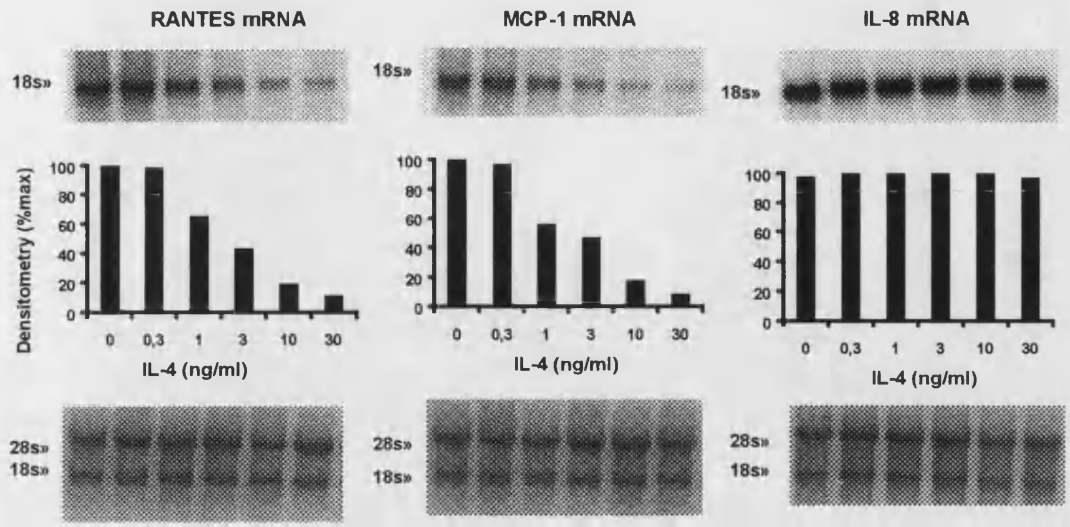

Figure 38 Effect of increasing concentrations of IL-4 on TNF-α/IFN-γ induced chemokine mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-4 and then TNF-α (100ng/ml)/IFN-γ (300 U/ml) were added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

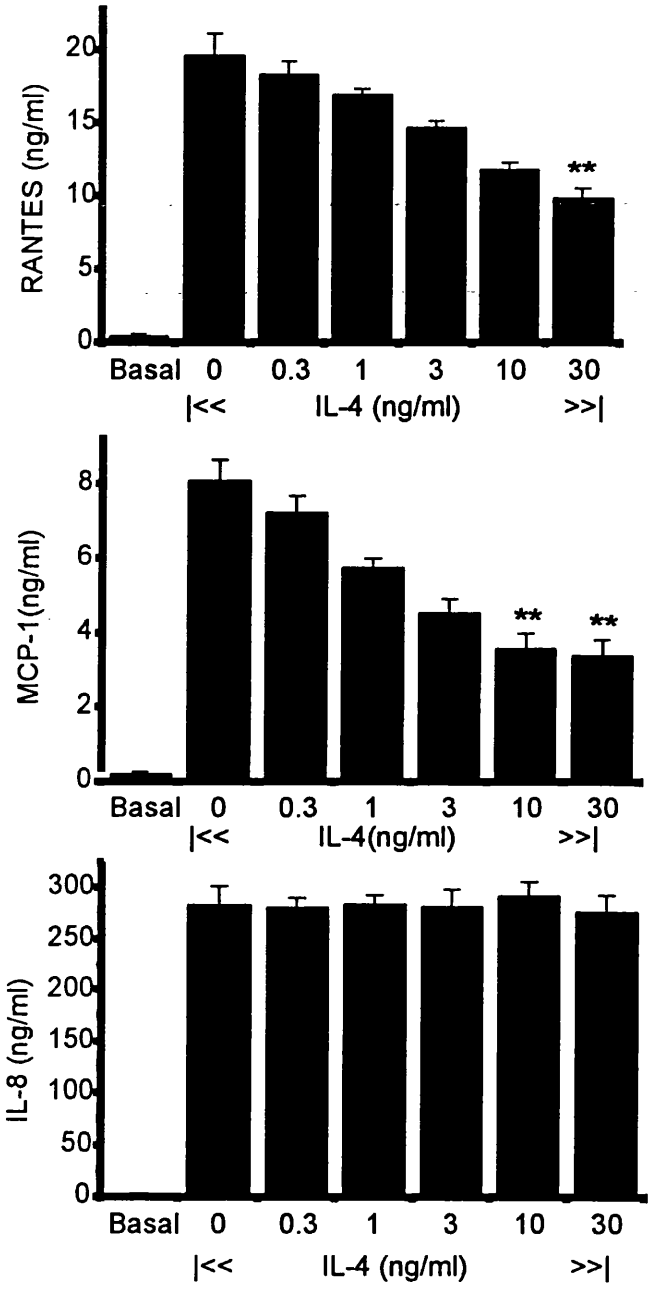

Figure 39 Effect of increasing concentrations of IL-4 on TNF-α/IFN-γ induced chemokine secretion by HT-29 cell line. Cells were pretreated for 1 hour with IL-13 and then TNF-α(100ng/ml)/IFN-γ(300 U/ml) were added. Each bar is the mean ± SEM of three experiments (** $p<0.01$).

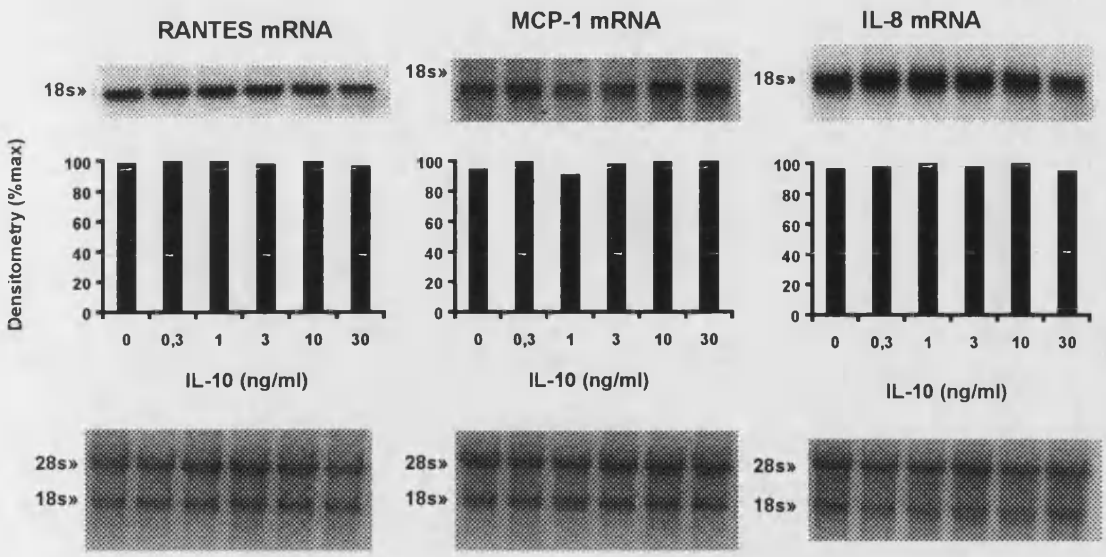

Figure 40 Effect of increasing concentrations of IL-10 on TNF-α/IFN-γ induced chemokine mRNA expression by HT-29 cell line. Cells were pretreated for 1 hour with IL-10 and then TNF-α (100ng/ml)/IFN-γ (300 U/ml) were added. The top panel is the northern blot, middle panel is densitometry analysis of blot and lower panel is the ethidium bromide stained 18s and 28s bands indicating equal loading of the lanes. Representative of three experiments.

from colon adenocarcinoma expressed heavy IL-8 staining in colon adenocarcinoma epithelial cells, while normal mucosa from adjacent areas were negative (Fig. 41D). No staining was seen in the negative controls, when the primary antibody was omitted (Fig. 41E).

3.2.6 Discussion

Intestinal inflammation is characterised by infiltration of lamina propria and epithelium by selected populations of leukocytes. The migration of leukocytes from the lumen of the microvasculature to distant extravascular sites is a characteristic of inflammatory diseases. A large family of target cell specific chemotactic polypeptides, collectively known as chemokines, are crucial in the orchestration of this leukocyte migration and activation (Miller & Krangel, 1992; Lindley *et al.* 1993). The study of chemokine expression in intestinal inflammation is still in its beginning and at the initiation of this study a small number of published information focusing on IL-8 was available.

We have clearly shown IL-8 expression using immunostaining in the colonic mucosa of patients with ulcerative colitis and infectious colitis, as well as in biopsies from colon adenocarcinoma. IL-8 expression was detected in the basal part of colonic epithelial cells in close association with neutrophil infiltration and in cells of the lamina propria. These results strongly suggest that the colonic epithelial cells are a major source of IL-8 production and that this production is associated with the intestinal inflammation. Similarly, the IL-8 production in colonic mucosa has been reported to be related with the grade of the intestinal inflammation (Mazzucchelli *et al.* 1994). These findings taken together suggest an involvement of colonic

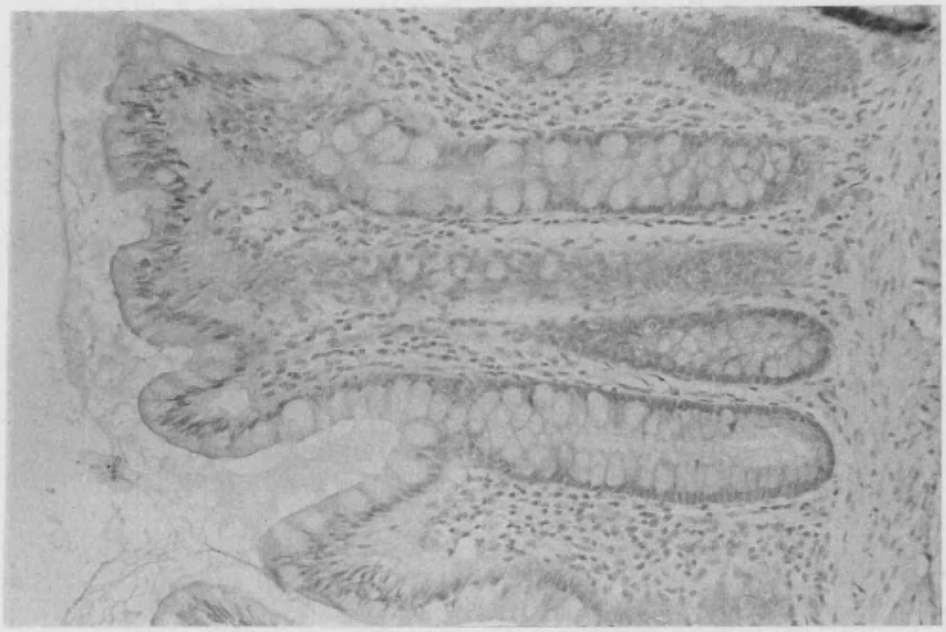

Figure 41A Normal colonic bowel mucosa with no detection of IL-8. Immunostaining with anti-human IL-8 antibody, using avidin-biotin peroxidase. Original magnification X 100.

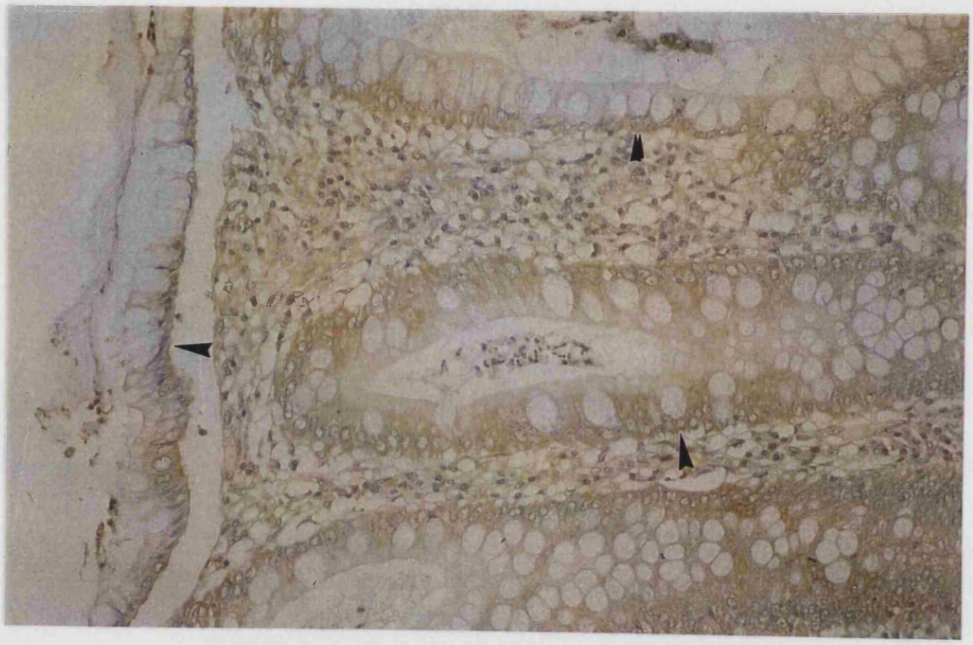

Figure 41B Ulcerative colitis with staining for IL-8. Colonic epithelial cells of superficial section of crypt and surface epithelium markedly express IL-8, which is expressed in basal part of the epithelium, in close association with the neutrophils of the lamina propria (arrows). Immunostaining with anti-human IL-8 antibody, using avidin-biotin peroxidase. Original magnification X 100.

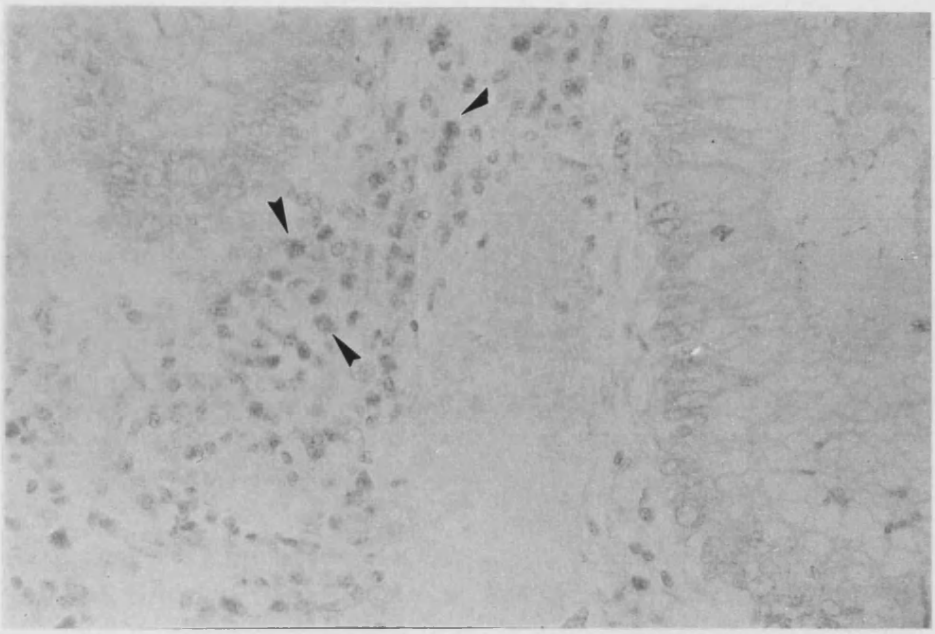

Figure 41C Another ulcerative colitis case showing numerous cells positive for IL-8, located at the lamina propria and epithelium (arrows). Immunostaining with anti-human IL-8 antibody, using avidin-biotin peroxidase. Original magnification X 250.

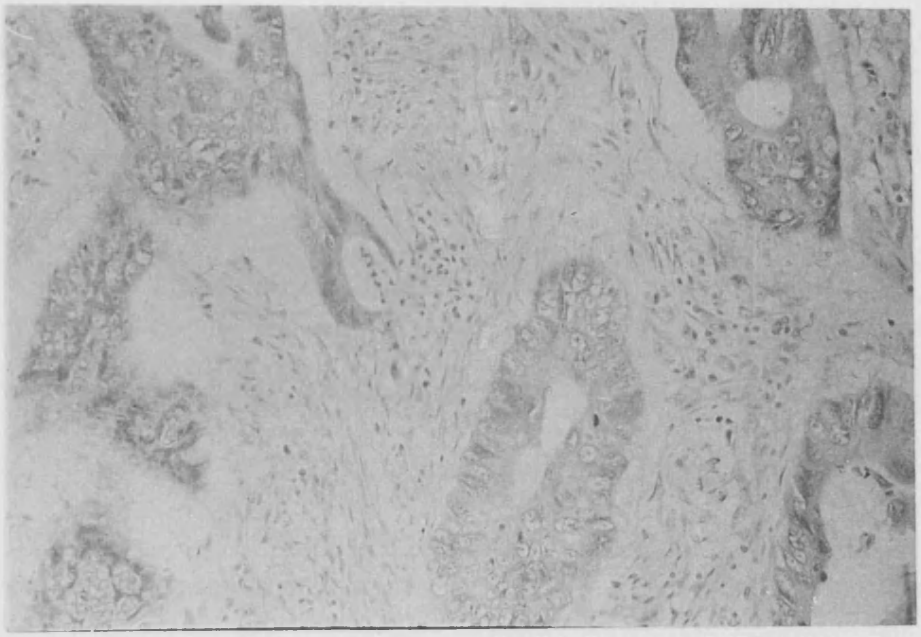

Figure 41D Biopsies from colon adenocarcinoma expressed heavy IL-8 staining in colon adenocarcinoma epithelial cells. Immunostaining with anti-human IL-8 antibody, using avidin-biotin peroxidase. Original magnification X 250.

Figure 41E Ulcerative colitis patient, negative control. No staining was seen, when the primary antibody was omitted. Original magnification X 50.

epithelial cells in leucocyte recruitment during the intestinal inflammation. Interestingly, in our study we have demonstrated for first time a heavy staining of colon adenocarcinoma cells in biopsies from colon adenocarcinoma. In marked contrast, in the same patients colonic epithelial cells from adjacent normal areas were negative for IL-8 expression. The latter observation suggest that colonic epithelial cells producing large amounts of IL-8 might be involved in tumour angiogenesis. While numerous investigations have shown both in vivo and in vitro the importance of IL-8 in acute inflammation, as a chemotactic / activating factor for neutrophils, recently has it become apparent that this C-X-C chemokine may be important in angiogenesis associated with tumorgenesis. IL-8 was found to be a potent angiogenic factor with similar angiogenic activity as basic fibroblast growth factor (bFGF)(Koch *et al.*, 1992).

In support of our findings from the immunostaining study of colonic biopsies we have found in vitro that the human colonic epithelial cells HT-29 in response to pro-inflammatory cytokines express IL-8 mRNA and produce large amounts of IL-8. Furthermore, we have demonstrated that these cells after induction with specific combinations of cytokines produce MCP-1 and RANTES, two members of C-C chemokine family. These results are in agreement with results published during the course of my work, demonstrating C-C chemokine expression in colonic mucosa during the intestinal inflammation (Reinecker *et al.*, 1995; Grimm *et al.*, 1996; Mazzucchelli *et al.*, 1996) and support the concept that colonic epithelial cells are involved in leukocyte migration. In our study IL-8 mRNA expression was observed as early as 1 hour, peaked at 4 hours and then rapidly declined. MCP-1 and RANTES mRNA expression showed a delay with a peak at 12 h and 24 h respectively. These finding are in step with the histopathology of colonic mucosa in the early acute phase of ulcerative colitis, which is

characterised by the presence of numerous neutrophils either infiltrating lamina propria or forming crypt abscesses and in the latter chronic phase, which is characterised by infiltration of the lamina propria with lymphocytes, plasma cells and eosinophils without neutrophils (Nostrant *et al.* 1987).

Examining the role of T cell derived cytokines on chemokine expression by colonic epithelial cells, we have demonstrated that IL-13 modulates chemokine expression by HT-29 cells. Thus epithelial cells must now be considered together with monocytes, endothelial cells, polymorphonuclear granulocytes and keratinocytes as targets for IL-13 (Zurawski & De Vries, 1994; Herbert *et al.* 1993; Derocq *et al.* 1994; Sironi *et al.* 1994; Colotta *et al.* 1994). This cytokine produced a doubling in the IL-8 secreted by epithelial cells that were stimulated by IL-1α, but was without effect on IL-8 generation in cells stimulated by TNF-α or TNF-α/IFN-γ. It was apparent at both the level of mRNA and peptide that the effect of IL-13 on chemokine production was restricted to IL-1α stimulated cells, as it was without effect on other cytokine stimulated cells. In addressing this unique action of IL-13 a number of observations are worth noting. Firstly, IL-8 production by HT-29 cells by supra-maximal concentrations of TNF-α or TNF-α/IFN-γ was approximately twice and three times, respectively, as much as that produced by supra-maximal concentrations of IL-1α, results similar to those reported previously (Schuerer-Maly *et al.* 1994). The relative potencies of IL-1α and TNF-α is the reverse of the pattern found in other cells and cell lines (Watson *et al.* 1988; Brown *et al.* 1991; Brown *et al.* 1994; Jordan *et al.* 1996). Secondly, the differential action of IL-1α and TNF-α or TNF-α/IFN-γ on chemokine production was also reflected at the level of IL-8 mRNA. Induction of IL-8 mRNA with IL-1α peaked at 1h, then rapidly subsided, in contrast, TNF-α or TNF-α/IFN-γ induced IL-8 mRNA expression similarly peaked by 1h, but was maintained for at least 12h. Thirdly, IL-13 caused a prolongation of IL-

1α induced IL-8 mRNA expression that was similar to that present in TNF-α or TNF-α/IFN-γ stimulated cells. Fourthly, experiments with cycloheximide, a protein synthesis inhibitor, demonstrated that the effect of IL-13 on IL-8 mRNA expression did not depend upon protein synthesis. Thus the action of IL-13 on epithelial cells is unlikely to involve IL-1ra because this would produce an inhibition of IL-1α action not an enhancement, and the involvement of IL-1ra is not consistent with the lack of effect of cycloheximide on IL-8 mRNA. Finally, experiments with actinomycin-D indicate that IL-13 is producing an enhanced IL-1α-induced IL-8 mRNA via an increase at transcription rather than mRNA stability.

Furthermore, we found that IL-13 in same concentrations had an inhibitory effect on MCP-1 and RANTES expression and secretion. This action of IL-13 on HT-29 epithelial cells is in marked contrast to the effect of IL-13 on monocytes, in which it is a potent inhibitor of cytokine and chemokine production (Minty *et al.* 1993; McKenzie *et al.* 1993; Zurawski & De Vries, 1994; De Waal Malefyt *et al.* 1993). The inhibitory effect of IL-13 on stimulated chemokine production by monocytes is probably via both the enhanced production of IL-1ra and soluble receptors, as well as a direct inhibition of chemokine transcription (Muzio *et al.* 1994). Similar results we found examining the effect of IL-4, which share many properties with IL-13 (Zurawski & De Vries, 1994). Both, IL-4 and IL-13 were found to have a differential effect on chemokine expression by colonic epithelial cells with an inhibitory effect on C-C chemokine expression, but an inflammatory effect inducing IL-8 production. Thus, when considering cytokine networks, particularly with respect to chemokine production, T cell derived cytokines can have a bifunctional role, depending upon the stimulus and the cellular source of chemokines. Finally, we found that IL-10 throughout the concentration range of 0.1 to 30 ng/ml had no effect on pro-inflammatory cytokine-induced chemokine production by the colonic epithelial cell line HT-29. Therefore the expression of "chronic

enterocolitis" phenotype in IL-10 deficient mice (Kahn *et al.* 1993) is probably not via a direct suppressive action of IL-10 on colonic epithelial cells.

Thus, this capacity of colonic epithelial cells to secrete chemokines, might be important in the inflammatory response, as leukocyte infiltration of the intestinal mucosa is a characteristic feature of the intestinal inflammation and an increased production of chemokines has been reported in colonic mucosa from patients with IBD (Mahida *et al.* 1992; Mitsuyama *et al.* 1994; Grimm *et al.* 1996; Mazzucchelli *et al.* 1996; Kolios *et al.* 1997). In addition, the findings that pro-inflammatory cytokines released by activated macrophages during an acute inflammatory response, induce chemokine production by colonic epithelial cells suggest a communication between these cells and immune cells located in the underlying intestinal mucosa. Production of the potent neutrophil and T-lymphocyte chemoattractant cytokines IL-8, MCP-1, and RANTES from intestinal epithelial cells may have a crucial effect on intra-epithelial and lamina propria neutrophils and lymphocytes during the intestinal inflammation. The infiltration of T-cells induced by chemokines could be particularly relevant to the role of colonic epithelial cells as antigen presenting cells (Mayer *et al.* 1991). Furthermore, our results that T-lymphocyte-derived cytokines IL-13 and IL-4 have a synergism with IL-1α in IL-8 secretion and expression by colonic epithelial cells support the view that there is an interaction between epithelial cells and lymphocytes in the initiation and maintenance of the intestinal inflammation.

4. DISCUSSION

The aetiology and pathogenesis of ulcerative colitis (UC) and Crohn's disease (CD), despite extensive investigations, is unclear and an abundance of data and theories implicate genetic predisposition, infectious agents or other environmental factors, and immunologic alterations, but the primary events, which initiate the inflammatory response remain unknown. Over the last years a great advance has been made in the understanding of the inflammatory process. Previously our knowledge was limited to a simple three-stage process. First, an irritant, immune or infectious agent activates leukocytes; second, these cells release enzymes and inflammatory mediators; and finally, these products cause pain, heat, oedema and loss of function (Zipser, 1988). We now know that the inflammatory process is more complicated and it is clear that mediators of inflammation also arise from cells other than leukocytes. Some of these mediators are involved in cell-cell communications, affecting the immune response, the synthesis and release of enzyme, and the cell proliferation and healing phase during the inflammation. The intestinal mucosal system depends on the co-operation of epithelial cells and lymphoid components to initiate and maintain an immunological response (Schmucker *et al.* 1996). In inflammatory bowel disease (IBD) the inflammation may be triggered by the entry of bacterial products or dietary antigens into the lamina propria through an epithelium with abnormal permeability, while environmental factors, such as infectious agents, may further stimulate the mucosal immune process, which is probably modified and propagated by a genetic predisposition. Regardless of the aetiology and the initiating event, the inflammatory response will be amplified through the release of soluble

mediators as well as the recruitment of cellular components of the immune system (Nielsen & Rask-Madsen, 1996).

During the last decade the traditional view of epithelial function has been expanded by the realisation that intestinal epithelium has a dual role in the control of intestinal physiology; the ability to serve simultaneously as both an absorptive surface and a barrier. Attention has been focused on the role of the epithelium in the initiation of chronic intestinal inflammation and two models have been proposed. First, a consistent deficiency in structural integrity may permit recognition of antigens, not normally encountered below the epithelium, which in the genetically susceptible host leads to inflammation; or second, epithelial elements, acting as transducers of normally non-pathogenic stimuli, promote and amplify inflammation, regardless of maintenance of normal structural integrity (Sands & Podolsky, 1996). The second model reflects the role of epithelial cells as an active participant in the immune response. Intestinal epithelial cells represent an important interface between the environment and the host tissue and in addition to their role as a selective barrier to prevent the entry of antigenic or infectious material into the body, they are involved in many inflammatory and immune reactions (McKay & Perdue, 1993a; McKay & Perdue, 1993b). Several observations suggest that the intestinal epithelial cells may serve as signal transducers, recognising antigens within the lumen and presenting information to underlying cell population within the lamina propria (Greenewald & James, 1995). Recently, it has been clear that colonic epithelial cells participate in the immune process producing a number of soluble inflammatory mediators. These cells present antigens via class II molecule expression (Mayer *et al.* 1991; Lowes *et al.* 1992), express adhesion proteins such as intercellular adhesion molecule-1 (Kaiserlian *et al.* 1991) and generate soluble

inflammatory mediators e.g. arachidonic acid derivatives (Gustafson & Tagesson, 1990; Dias *et al.* 1992), platelet activating factor (Ferraris *et al.* 1993), and cytokines (Hedges *et al.* 1992; Eckmann *et al.* 1993; Schuerer-Maly *et al.* 1994; Gross *et al.* 1995; Jung *et al.* 1995), all of which contribute to the communication between inflammatory cells and cells of the immune system (Sartor, 1994). The purpose of our study was to examine the role of colonic epithelial cells in the production and release of inflammatory mediators during the intestinal inflammation and to define which agents modulate this process. To attain these goals our study has focused on the production of nitric oxide (NO) via the expression and activation of the inducible nitric oxide synthase (iNOS) and the involvement of the colonic epithelial cells in leukocyte recruitment in the site of the inflammation via the expression and secretion of chemokines.

In our study, we have demonstrated that human HT-29 colonic epithelial cells in response to specific combinations of cytokines express iNOS mRNA and produce large quantities of nitrite. Tissue-derived cytokines are known to be potent inducers of NO synthase in macrophages, neutrophils and endothelial cells but the demonstration of such activity in human colonic epithelial cells is potentially of great importance in colonic inflammation. Pro-inflammatory cytokines were found increased in intestinal inflammation and a crucial role of these mediators has been proposed in IBD (Kolios & Nakos, 1995; Sartor, 1994; Radford-Smith & Jewell, 1996). The observation that these cytokines stimulate HT-29 cells to produce large amounts of NO suggests an implication of colonic epithelial cells in immune reactions during the intestinal inflammation. Although our results were obtained with a colonic epithelial cell line, which has many characteristics in common with "normal" colonic epithelial cells

(Chantret *et al.* 1988) the possibility exists that "normal" cells may behave differently to tumour cells. Studies in patients with UC have shown a remarkable increase in NO synthase activity in the inflamed mucosa compared to the uninflamed controls (Middleton *et al.* 1993a; Boughton-Smith *et al.* 1993). Nitrite, the stable end product of NO production was found in measurable amounts in rectal dialycates from patients with active UC (Eckmann *et al.* 1993) and luminal NO was greatly increased in UC patients (Lundberg *et al.* 1994a). They proposed that luminal NO measurement may reflect NO production in the most superficial parts of the mucosa, since NO produced in deeper mucosal layers is probably trapped (e.g., by haemoglobulin in mucosal blood vessels) and therefore will not reach the lumen, suggesting that the excess NO production in UC mainly occurs in very superficial mucosal layers (Lundberg *et al.* 1994a). However, the cellular source of NO in the intestinal mucosa was not identified.

To support our *in vitro* findings, an immunohistochemical study of colonic biopsies was performed, which demonstrated intense iNOS staining in the inflamed colonic epithelium of patients with UC and infectious colitis. The colonic epithelial cells that expressed iNOS occurred in sites of inflammation and were associated with areas of intense neutrophil infiltration of lamina propria and epithelium. Uninflamed mucosa was negative for iNOS labelling. Singer et al. working in parallel, using the same antibodies found similar results. Furthermore, in the same cases which expressed iNOS, they found that nitrotyrosine, the stable product of the action of peroxynitrite on tyrosine-containing proteins, was also localised in colonic epithelial cells in areas of inflammation, both in the surface epithelium and crypts (Singer *et al.* 1996). These findings suggest that some components of the inflammatory

response induces iNOS expression in colonic epithelial cells and that NO generated by these cells reacts with superoxide to produce peroxynitrite, which nitrates cellular proteins and forms nitrotyrosine. Whether the nitration of tyrosine is associated with functional impairment of the colonic epithelium is not known, but these findings suggest an implication of colonic epithelial cells in the NO-dependent nitrosation reactions during the intestinal inflammation. Aminosalicylates, which are widely used to treat IBD, inhibit these nitrosation reactions; (Grisham & Miles, 1994) this activity may account for their therapeutic effects in intestinal inflammation.

The production of NO by colonic epithelial cells is also supported by similar results in other studies. Calcium-dependent NO synthase activity was previously shown to be present in gastric epithelial cells (Brown *et al.* 1992), and also in epithelial cells isolated from rat proximal small intestine (Tepperman *et al.* 1993). Using in situ hybridisation and immunohistochemistry to detect iNOS, they have demonstrated high expression of iNOS localised to the surface epithelium and crypts in the mucosa from patients with UC (Reynolds *et al.* 1995). iNOS was immunolocalised in ileal epithelial cells of lipopolysaccharide treated rats but not in controls (Cook *et al.* 1994), in addition rat intestinal epithelial cells in culture when stimulated with cytokines and lipopolysaccharide were found to produce NO (Grisham, 1993). NO synthesis has also been found in epithelial cells in rhesus monkeys with idiopathic colitis, but not in control monkeys (Ribbons *et al.* 1995). Induction of iNOS expression is not limited to intestinal epithelial cells as it has been identified in rodent and human epithelial cells *in vivo* and *in vitro* (Robbins *et al.* 1994b; Robbins *et al.* 1994a; Gutierrez *et al.* 1995). Although in

our study iNOS expression was absent in normal mucosa, they have detected iNOS mRNA in normal human airway epithelium (Guo *et al.* 1995).

The functional role of the NO production by the colonic epithelial cells is not clear. Increased expression and production of NO may explain some of the pathophysiological features of active IBD. UC and CD are associated with mucosal vasodilation and increased vascular permeability, which result in mucosal erythema and oedema. IBD is also associated with enhanced epithelial permeability (Bjarnason *et al.* 1995). NO is a potent vasodilator and it is known that during active episodes of colonic inflammation blood flow to the mucosa and submucosa of the colon is increased between to 2 and 6 fold (Hulten *et al.* 1977). NO may be an important pathway whereby inflammation induces hyperaemia, mucosal vasodilatation and increased vascular permeability (Middleton *et al.* 1993a). While an increase in blood flow alone would not cause an increase in vascular permeability, it would enhance the actions of other inflammatory mediators, which have a direct injurious action on the microvascular endothelium (Whittle, 1995). Diarrhoea is a main symptom of patients with IBD; this symptom is multifactorial, but usually has a secretory component. NO is important in the control of absorption from intestine. At physiological concentrations NO promotes absorption but at higher levels may produce secretory effects in the colon (MacNaughton, 1993). Furthermore, NO is capable of affecting alterations in intestinal motility in IBD by mediating the effects of gastrointestinal hormones and non-adrenergic and non-cholinergic mediated relaxation of colonic smooth muscle (Hata *et al.* 1990). In addition, NO relaxes colonic circular smooth muscle even in low concentrations (Middleton *et al.* 1993b). In active UC this can contribute to diarrhoea by depressing segmentation in the colon and in fulminant colitis impaired colonic

motility is associated with toxic megacolon, which may lead to perforation (Boughton-Smith, 1994). It has been shown that NO relaxes the human internal anal sphincter (Burleigh, 1992). Sufficient amounts of NO released by colonic epithelial cells may diffuse to the underlying muscle layer, in a similar way to the diffusion of NO from endothelium to the vascular smooth muscle (Moncada *et al.* 1991), and hence could affect the sphincter function and contribute to the urgency to stool, a frequent symptom in patients with UC. NO produced by colonic epithelial cells may therefore contribute to all these symptoms of IBD through a local action to colonic epithelium and affecting simultaneously the deeper layers of the bowel.

It is not clear whether the increased NO production in patients with IBD is beneficial or destructive for the tissue. Although NO or subsequent reactive products can contribute to cytopathology against host cells when produced in excess (Tepperman *et al.* 1994), some data suggest that NO may diminish epithelial damage. NO can scavenge oxygen radicals reducing their harmful effects (Grisham, 1994) and inhibition of NO synthesis promotes damage induced by HCl, ethanol, and lipopolysaccharide (Kitagawa *et al.* 1990; MacNaughton *et al.* 1989; Hutcheson *et al.* 1990) suggesting that NO has a protective effect on the gut. Epithelial iNOS expression could provide an oxidative barrier to bacterial invasion. This idea is supported by the findings that patients with active UC have lower numbers of bacteria in the rectal mucosa than patients in remission or normal controls (Hartley *et al.* 1992). Furthermore, large concentrations of NO are normally present in the nasal airways and the stomach without causing local tissue damage (Lundberg *et al.* 1994c; Lundberg *et al.* 1994b). These results suggest that NO production may contribute to repair of the epithelial barrier in some acute conditions. On the other hand, enhanced NO release via the induction and activation of iNOS

in the colon may contribute directly to the mucosal damage by cytotoxic activity by a variety of mechanisms including inhibition of DNA synthesis, inhibition of mitochondrial function and intracellular iron release (Kwon *et al.* 1991; Drapier & Hibbs, 1986). NO may cause tissue damage (McCall *et al.* 1989), freely interact with oxygen metabolites to yield nitrosating species and the formation of carcinogenic nitrosamines, shown to be generated by neutrophils during intestinal inflammation (Grisham *et al.* 1992), may be the link with the increased frequency of colorectal cancer in UC. High levels of nitrosamines have been demonstrated in rectal dialysates of patients with active IBD (MacNaughton, 1993). In our study unstimulated HT-29 colonic epithelial cells were found to produce a basal amount of constitutive NO, while stimulation with cytokines induced a large amount of NO via iNOS expression. It is possible that small amounts of NO produced in colonic epithelial cells by constitutive NOS may act to preserve intestinal integrity, while the large amounts of NO produced via iNOS induction have a proinflammatory effect causing tissue injury.

In conclusion we have shown that human colonic epithelial cells express iNOS activity during the intestinal inflammation. This expression of human iNOS mRNA and nitrite generation in colonic epithelial cells, *in vitro*, is differently regulated by combinations of cytokines. If these results can be extrapolated to primary colonic epithelial cells then the hypothesis that NO and its metabolites are derived from epithelial cells may be of central pathogenic significance in intestinal inflammation. These results have stimulated us to search for naturally occurring agents, such as asymmetrical dimethylarginine which is found in renal failure (Vallance *et al.* 1992) and pharmacological agents that might inhibit the production of active nitrogen species and prove of value in the treatment of IBD. However, the use of NO generation inhibitors

should proceed with caution. In UC, where increased NO synthase may contribute to vasodilatation, increase vascular permeability and damage of mucosal integrity, inhibitors of NO synthesis may be beneficial. In contrast, induction of NO in intestinal inflammation may in part serve to maintain colonic microvascular perfusion and may counteract the increased synthesis of vasoconstrictors and prevent microvascular coagulation by inhibiting platelet aggregation and adhesion (Boughton-Smith, 1994). Therefore, the use of NOS inhibitors could produce a NO deficiency in IBD causing a change in the pathophysiology of the disease. Whether the inhibition of NO production will be of therapeutic value in intestinal inflammation awaits further investigation of NO synthase isoforms and their differential induction, and probably the development of selective NOS inhibitors in order to inhibit the excessive NO production by the inducible NOS, without changing the physiological control of vascular tone and cellular integrity by the constitutive enzyme (Whittle, 1994).

The transmigration of selected populations of leukocytes is a multiple-step process and requires a series of co-ordinated signals, which include the expression and activation of adhesion molecules as well as the generation of a leukocyte specific chemotactic gradient by the cells of the extravascular component (Carlos & Harlan, 1994). However, the nature of the stimulus and the subsequent spectrum of chemotactic factors produced, determine the specific leukocyte population elicited to the inflammatory site (Baggiolini *et al.* 1994). This hypothesis is supported by the characterisation of a large family of target cells specific chemotactic polypeptides, now collectively known as chemokines (Lindley *et al.* 1993a; Lindley *et al.* 1993b). The IBD is characterised by the accumulation of inflammatory cells in the lamina propria and the infiltrate is composed predominantly of neutrophils, lymphocytes,

monocytes/macrophages, and plasma cells. Several studies have documented the recruitment of neutrophils (Saverymuttu *et al.* 1986) and monocytes (Grimm *et al.* 1995) from the circulation to the sites of active intestinal inflammation. Leukocyte adhesion, migration and tissue infiltration is critical to the development of intestinal inflammation, thus an increasing interest exists in understanding the precise mechanisms involving chemokines, epithelial cells, and circulating leukocytes that are implicated in this process (Kam *et al.* 1995). To contribute to this hypothesis we examined the expression of chemokines in colonic epithelial cells and their modulation by cytokines. In immunohistochemical studies of colonic biopsies from patients with UC and infectious colitis we have clearly shown IL-8 labelling in colonic epithelial cells in close association with sites of inflammation. In the same study IL-8 expression was detected in colon adenocarcinoma cells from colon adenocarcinoma biopsies. *In vitro*, the study of chemokine expression by colonic epithelial cells revealed a marked production of IL-8 after stimulation with pro-inflammatory cytokines, added alone or in combination. In contrast, the minimal requirement for the induction of the C-C chemokines, MCP-1 and RANTES was the combination of TNF-α and IFN-γ, while no other cytokine or pair of cytokines were capable of producing these monocyte chemoattractant cytokines.

Similar results from other studies support our findings that colonic epithelial cells express chemokines and they are involved in the leucocyte recruitment during the inflammatory process. Elevated IL-8 levels in inflamed mucosa from IBD patients were found in colonic biopsy specimens and this increased was correlated with grade of inflammation, number of neutrophils, tissue levels of IL-1α and TNF-α (Mitsuyama *et al.* 1994; Mazzucchelli *et al.* 1994), and macroscopic inflammation (Daig *et al.* 1996). IL-8 has been recognised as an

important chemoattractant for neutrophils in UC and CD (Raab *et al.* 1993; Mitsuyama *et al.* 1994). Several studies have strongly implicated the colonic epithelium as an important source of IL-8 in gut mucosa (Gibson & Rosella, 1995). Colonic epithelial cell lines and freshly isolated human intestinal epithelial cells were found to express IL-8 following stimulation by inflammatory cytokines and LPS (Eckmann *et al.* 1993; Schuerer-Maly *et al.* 1994). This IL-8 induction by cytokines was independent from protein kinase A, did not require protein kinase C, but involved protein tyrosine kinase activity (Gross *et al.* 1995). Our results from the immunostaining study of colonic biopsies that colonic epithelial cells from uninvolved and normal bowel mucosa did not expressed IL-8, while specimens from inflamed mucosa of UC and infectious colitis were positive for IL-8, are in parallel with observation from other studies (Mazzucchelli *et al.* 1994), suggesting an important but not specific role for this chemokine in IBD.

In addition, in biopsies from patients with colon carcinoma we detected IL-8 expression in colonic adenocarcinoma cells. IL-8 was found to stimulate proliferation and it has been suggested that, while IL-8 may have a role in attracting tumour-infiltrating leukocytes, colonic epithelial tumours may also derive a proliferative advantage from the capacity to generate IL-8 (Schuerer-Maly *et al.* 1994). The capacity of colonic epithelial cells to secrete IL-8 may be important in several respects. Firstly Strieter et al. in a series of papers have demonstrated that ELR expressing CXC chemokines are potent angiongenic factors expressed by tumour cells and that inhibition of these leads to suppression of tumour growth, conversely non ELR expressing CXC chemokines are angiostatic and inhibition of these leads to increased tumour growth (Strieter *et al.* 1995b; Strieter *et al.* 1995a; Koch *et al.* 1992). They are the first host

cells that come in contact with luminal bacteria and therefore they function as an early host signalling system to the immune cells located in the underlying intestinal mucosa. Bacterial invasion of intestinal epithelial cells has been found to induce IL-8 secretion by these cells (Eckmann *et al.* 1993). Colonic epithelial cell-derived IL-8 could initiate events recruiting leukocytes and leading to acute inflammation, in response to invasion by luminal pathogens. In addition, secretion of inflammatory cytokines and IL-8 by other cell types, including macrophages (Friedland *et al.* 1992; Koch *et al.* 1992) and neutrophils (McCain *et al.* 1993), could amplify an ongoing acute immune response leading to chronicity. This hypothesis is consistent with our findings that IL-1α and TNF-α, cytokines predominantly released by activated macrophages during an acute inflammatory response, increase significantly IL-8 production by colonic epithelial cells. Intestinal epithelial cells have the capacity to express, in addition to IL-8, a number of important mediators of an acute inflammatory response, including IL-1α, IL-1β, and TNF-α (Eckmann *et al.* 1993). These cytokines are also known to be produced by macrophages following LPS activation or active phagocytosis (Friedland *et al.* 1992). The similarity of cytokines that can be expressed by colonic epithelial cells and macrophages suggests that they may share common functions and it may be that both cell types are a part of a non antigen specific immune system that can recognise and respond to a limited but important number of signals (Eckmann *et al.* 1993; Friedland *et al.* 1992).

Other chemokines that have been studied in IBD include gro-α, MCP-1, RANTES, MIP-1α and eotaxin. Gro-α levels were found significantly raised in active UC both compared to the normal control group and to active CD (Isaacs *et al.* 1992). MCP-1 was detected elevated in macrophages, smooth muscle cells and endothelial cells, but not in epithelial cells in active

IBD and they demonstrated that therapeutic agents, as 5-aminosalysilic acid and cyclosporin A, reduced MCP-1 production (Grimm *et al.* 1996). In contrast, other studies have revealed MCP-1 expression in human colonic epithelial cells (Franci *et al.* 1995; Jung *et al.* 1995; Rogler *et al.* 1997; Kucharzik *et al.* 1997). Reinecker et al (1995) detected that intestinal epithelial cells also express MCP-1 constituitively and that this chemokine expression is increased by IL-1β stimulation. Constituitively MCP-1 expression could contribute to the attraction of lamina propria macrophages, which are commonly found in high numbers just beneath the epithelial layer (Mahida *et al.* 1989). Increased MCP-1 expression by epithelial cells may not only lead to the increased migration of macrophages into the lamina propria, but could also result in the activation of resident macrophages (Rollins *et al.* 1991). The secretion of a variety of injurious molecules from activated macrophages could cause epithelial cells damage. MCP-1 could also stimulate increased IL-1β production by monocytes (Jiang *et al.* 1992), which could create a circuit that would lead to a chronicity of inflammation within the intestinal mucosa. RANTES, another monocyte chemoattractant cytokine was found expressed in human intestinal mucosa in IBD (Mazzucchelli *et al.* 1996) and rotavirus infection induced increased RANTES production in intestinal epithelial cells (Casola *et al.* 1997). The increased RANTES expression in the intestinal mucosa of patients with IBD is of special interest, since the RANTES-mediated chemotactic activity for CD4+ "memory" T-lymphocytes (Schall *et al.* 1990; Proost *et al.* 1996) may lead to up-regulation of mucosal immune responses and may exacerbate chronic inflammation. Data that have shown MCP-1 and RANTES mRNA expression in intestinal mucosa of control patients indicate that local expression of these two chemokines is not specific for IBD, but may allow the continued recruitment of immune cells responsible for an efficient immunological defence system in an anatomical site with a high antigenic presence

(Mazzucchelli *et al.* 1996). The CC chemokines are not only involved in recruitment of T cells, but are also activators of T cells e.g. IL-2 receptor expression (Taub *et al.* 1995b; Turner *et al.* 1996). Finally, intestinal epithelial cells were also found to express eotaxin (Lu *et al.* 1997), MIP-1α (Rogler *et al.* 1997), and gro- α (Casola *et al.* 1997).

Furthermore, epithelial cells from other organs, including skin (Barker *et al.* 1991; Gillitzer *et al.* 1991), lung (Standiford *et al.* 1990), kidney (Schmouder *et al.* 1992), and bladder were found to produce IL-8. Gro and MCP-1 is induced by inflammatory cytokines in human retinal pigmented epithelium (Stadnyk, 1994), and human renal cortical epithelial cells are a source of MCP-1 production (Schmouder *et al.* 1993). It is now clear that epithelial cells respond to infection or injury with chemokine secretion and serve as an early signal in immune and inflammatory reactions. These data suggest that a bi-directional communication exists between colonic epithelial cells and mucosal immune and inflammatory cells, which may be of paramount importance in gut mucosal defence.

There is a strong evidence that intestinal T-lymphocytes play crucial regulatory role in local mucosal immunity. Once absorbed, luminal antigens are processed and presented on the surface of antigen presenting cells in association with histocompatibility leukocyte antigen (HLA) class II molecules. Then, the processed antigen is recognized by antigen sensitive CD4+ cells resulting in their immune-activation and secretion of IL-2 and IFN-γ, which induce CD4 cells to undergo clonal expansion into memory and effector cells. Furthermore, IFN-γ induces MHC class II expression on antigen presenting cells (APCs) and cytokine secretion from macrophages that continues to amplify the immune response (Przemioslo & Ciclitira, 1996).

T(helper)-cells have been designated Th1 and Th2 according to the pattern of cytokines they secrete (Mosmann & Coffman, 1989). Gross regulation of Th1 and Th2 lymphocyte subsets may provide a basis for the mutually exclusive humoral and cell mediated response to antigen and cytokines released by one sub-group of T cells can down-regulate the secretion of cytokines from the other (Fiorentino *et al.* 1989). A balance between the predominantly inflammatory Th1 cytokine response and the more protective or humoral Th2 response is thought to play an important role in gut immune responses (Godkin *et al.* 1996). Recent data by Lukacs et al. indicate that CC chemokines may regulate the Th1/Th2 balance and thus regulate the immune responce (Karpus *et al.* 1997). Some differences observed in the repertoire of T lymphocytes in gut of UC and CD patients suggest that they play a role controlling immunity into the intestinal mucosa in IBD (Greenewald & James, 1995). The concept that IBD is mediated by Th1 and Th2 lymphokines is further supported by observations of increased tissue concentrations of IL-4 and IL-10 in UC but not in patients with CD (Sartor, 1994; Kolios & Nakos, 1995).

Intra-epithelial leukocytes have been reported in intestinal mucosa and these are a prominent characteristic of intestinal inflammation. Although their role remains uncertain it is probable that they are involved in a spectrum of immunological events within intestinal mucosa (Croitoru & Ernest, 1992; Cerf-Benussan & Gay-Grand, 1991). T-lymphocyte accumulation in lamina propria and infiltration of the colonic epithelium by these cells is a characteristic feature of the intestinal inflammation (Croitoru & Ernest, 1992; Cerf-Benussan & Gay-Grand, 1991). Intestinal epithelial cells take part in the mucosal immune response and play a role in antigen presentation to mucosal T cells (Shanahan, 1993; Greenewald & James, 1995) through their

capacity to express HLA class II antigens (Mayer *et al.* 1991). Expression of class II molecules was found increased on epithelial cells located near lymphoid follicles, which are increased in IBD (Chiba *et al.* 1994) and a disturbance in lymphoepithelial communication has been proposed in IBD (Shanahan, 1993).

To explore the role of T-cell derived cytokines in the regulation of the production of inflammatory mediators by colonic epithelial cells we studied three Th2 derived cytokines, namely IL-13, IL-4 and IL-10, that have been proposed as anti-inflammatory agents (Minty *et al.* 1993; Zurawski & De Vries, 1994; Moore *et al.* 1993). In our study we examined, *in vitro*, the regulatory effect of Th2 derived cytokines on the iNOS and chemokine expression and activity induced by pro-inflammatory cytokine in the colonic epithelial cells HT-29. In addition we studied the effect of IL-13 on the cytokine induced nitrite production in cultures of human colonic mucosa biopsies. IL-13 and IL-4 were found to modulate iNOS as well as chemokine expression and activity in colonic epithelial cells. In marked contrast IL-10 had no any effect. IL-13 was found to inhibit significantly the IL-1α/IFN-γ and IL-1α/IFN-γ/TNF-α induced iNOS expression and activity by HT-29 cells. In addition IL-13 significantly reduced the nitrite production in cultures of colonic biopsies treated with "cocktail" of cytokines. IL-4 shared with IL-13 the inhibitory effect on iNOS expression and activity by activated HT-29 cells, but in marked contrast, it was not found to have the up-regulating effect of IL-13 on IL-1α/IFN-γ-induced iNOS expression and activity. Similar, but independent biological functions of IL-4 and IL-13 suggest that IL-4 and IL-13 receptors are closely related but distinct (Callard *et al.* 1996). Interestingly, the latter observation in our present study suggest that these two cytokines share some, but not all, of their biological activities in the same type of cells. Differences have

been reported in signal transduction by IL-4 and IL-13 (Keegan *et al.* 1995) that could explain our findings. Both IL-4 and IL-13 markedly inhibited MCP-1 and RANTES expression and secretion by HT-29 cells, but they had no effect on IL-8 production by colonic epithelial cytokines treated with combination of pro-inflammatory cytokines. Similarly in other studies, IL-4 and IL-13 were found to down-regulate MCP-1 expression in activated intestinal epithelial cells (Kucharzik *et al.* 1997), while lack of inhibition of IL-8 production in epithelial cell lines was detected after stimulation with the T cell derived cytokines IL-4 and IL-10 (Schuerer-Maly *et al.* 1994).

The inhibitory effect of Th2 cell derived cytokines IL-4 and IL-13, we found, is in parallel with findings from other studies. For example IL-13 is a potent suppressor of cytokine and chemokine expression by activated monocytes and macrophages (Minty *et al.* 1993; McKenzie *et al.* 1993; Zurawski & De Vries, 1994; De Waal Malefyt *et al.* 1993), and endothelial cells (Marfaing-Koka *et al.* 1995). In addition IL-13 and IL-4 inhibit iNOS expression in human messangial cells, but IL-13 was suggested as a potential therapeutic tool, because it shows a wider temporal window of intervention (Saura *et al.* 1996). Indeed, we have recently demonstrated that the mechanism of IL-13-induced inhibition of iNOS is via the activation of phosphatidylinositol 3-kinase (Wright *et al.* 1997). Both IL-4 and IL-13 are considered as anti-inflammatory cytokines. However, both of these cytokines also have a pro-inflammatory effect because each enhances B cell production of IgE (Defrance *et al.* 1994). In our study, for first time IL-13 was found to express a new inflammatory property. It was found to enhance the IL-1α-induced IL-8 expression and secretion in HT-29 cells, and IL-4 was detected to have the

same effect on HT-29 cells (Dr Adrian Minty, personal communication), suggesting a bifunctional effect of these cytokines on the colonic epithelial cells.

Interleukin-10 is a 35 kDa homodimeric cytokine, which was originally described as Th2-cell-derived cytokine. Recent studies have shown that IL-10 is produced by a variety of cells, including Th2 cells, Ly-1 B cells, mast cells and cells of the macrophage lineage (Moore *et al.* 1993). This cytokine at concentrations of 10 ng/ml or less is an effective inhibitor of cytokine generation by IFN-γ and/or LPS stimulated human monocytes (De Waal Malefyt *et al.* 1991), prostaglandin E_2 production by IL-1 or LPS activated human monocytes (Poole *et al.* 1995) and chemokine generation by polymorphonuclear leukocytes treated with LPS (Kasama *et al.* 1994). IL-10 has been found to inhibit NO production by mouse activated macrophages (Oswald *et al.* 1992; Cenci *et al.* 1993). Thus IL-10 is considered to be a modulator of the inflammatory response. However, we found that IL-10 throughout the concentration range of 0.1 to 30 ng/ml had no effect on pro-inflammatory cytokine-induced iNOS expression and activity by the colonic epithelial cell line HT-29. Similarly, IL-10 had no any effect on chemokine generation by HT-29 cells, in marked contrast with the other T cell derived cytokines, IL-4 and IL-10. Thus, the expression of "chronic enterocolitis" phenotype in IL-10 deficient mice (Kahn *et al.* 1993) is probably not via the loss of a direct suppressive action of IL-10 on colonic epithelial cells, but is probably due to the loss of the suppresive action of IL-10 on T cells and monocytes. IL-10 was found to down regulate effectively the enhanced secretion as well as mRNA levels of IL-1β and TNF-α by IBD peripheral monocytes and intestinal lamina propria mononuclear cells. In addition, IL-10 was capable of inducing IL-1ra secretion in both peripheral monocytes and intestinal lamina propria mononuclear cells and

restoring the decreased IL-1ra/IL-1β ratio in IBD to normal levels (Schreiber *et al.* 1995b). This protective effect of IL-10 was found transitory, suggesting that IL-10 was able to down-regulate the local inflammatory reactions by inhibiting the synthesis if inflammatory cytokines, but did not reverse the inflammatory potential of the immune cells (Goldman *et al.* 1997). Therefore, primary defect in IL-10 expression may be a failure to control normal intestinal immune responses, leading to chronic inflammation via continuous overproduction of pro-inflammatory cytokines such as IL-1 and TNF-α (Kahn *et al.* 1993). These findings propose that IL-10, because of its ability to inhibit monocyte/macrophage activation it may be a useful therapy in IBD (Schreiber *et al.* 1995b; Duchmann *et al.* 1996).

In conclusion we found, that colonic epithelial cells produce a spectrum of inflammatory mediators, depending on the stimuli and the duration of the stimulation, as they expressed for example an early signal for IL-8 and a delay expression of C-C chemokines and NO production. In addition T cell derived cytokines were found to have a differential effect on the colonic epithelial cells activation. Taken together our results suggest that T cell derived cytokines can have a bifunctional role in the regulation of inflammatory mediators by colonic epithelial cells, depending upon the stimulus and probably the phase of the disease. This differential effect of T cells on the colonic epithelial cell inflammatory response, via a cytokine network, could play an important role in the inflammatory process and define the remission or the chronicity of the intestinal inflammation.

Studies of the migration of human intestinal intraepithelial lymphocytes (IEL) have shown that IELs first are attracted by chemokines in the lamina propria and then by chemokines in the

epithelium (Ebert, 1995). Colonic epithelial cells producing chemokines, as MCP-1, RANTES, and IL-8, in response to cytokine activation and bacterial invasion are capable of drawing IELs to the epithelium. RANTES with its chemotactic activity for CD4+/CD45RO+ 'memory' T-lymphocytes (Schall *et al.* 1990) and MCP-1 with its property of T lymphocyte chemoattraction (Carr *et al.* 1994; Taub *et al.* 1995a) contribute in T lymphocyte migration into the epithelium. In addition, IL-8, originally described as a neutrophil chemoattractant cytokine, has been shown to attract T lymphocytes (Larsen *et al.* 1989). IL-8 and RANTES were found to be the most attracting cytokines in intestinal epithelium, attracting large fractions of IELs, mainly because of chemotaxis rather than chemokinesis (Ebert, 1995). Thus, our *in vitro* results suggest that colonic epithelial cells producing chemokines are involved in T cells recruitment and participate in a bi-directional communication with T lymphocytes. An imbalance in this communication has been suggested to be implicated in pathogenesis of IBD (Schreiber *et al.* 1995a; West *et al.* 1996). For example, differences of IL-4 and IL-10 production in CD compared to UC have been considered responsible for the failure to detect induction of iNOS in CD (Boughton-Smith *et al.* 1993; Boughton-Smith, 1994) and therapeutic approach with T cell derived cytokines has been proposed (Kucharzik *et al.* 1996). It will be important to determine whether colonic epithelial cells from normal individuals and from patients with IBD also respond to the same stimuli. This might provide useful information on the leukocyte recruitment in the colonic epithelium during intestinal inflammation and help with the understanding of the pathogenesis of IBD. *In vivo* it is likely that the optimal cocktail of inflammatory cytokines is present in gut epithelium and capable of inducing chemokine and iNOS activity by colonic epithelial cells. Strategies to increase apical epithelial concentrations of T cell derived cytokines could provide an anti-inflammatory approach to intestinal

inflammation. Alternatively identification of IL-4, IL-10, and IL-13 signal transduction system responsible for the common inhibitory effect of these cytokines on inflammatory mediators expression should reveal novel targets for therapeutic intervention of IBD.

The mechanisms of inflammation represent an obscure network of cytokine-immune cell interactions, which are under a constant regulation, and a dynamic balance must exist in order to maintain an harmony between the ability to respond to noxious antigens or stimuli, while at the same time to limit and down-regulate this cascade of reactions to prevent it from producing excessive injury (McCarthy, 1994). Recent studies support the hypothesis that the pathogenesis of IBD involves a dysfunction of the intestinal immune system due to an imbalance between activating and suppressing cellular mediators, which results in a chronic inflammation (Kam *et al.* 1995). The intestinal mucosa is composed of several cell population, among them epithelial cells, immune cell populations and fibroblasts. It is presumed that complex mechanisms exist to maintain an homeostasis and simultaneously to co-ordinate an interaction between these cell populations. Nevetherless, it is clear that a intricate network of soluble mediators is essential in regulating the cascade of reactions from the recognition of the stimuli to the inflammatory process. The intestinal epithelial cells constitute a barrier between the environment and the host tissue and they are the first cells to come in contact with many pathogens, thus when invaded by external antigens can function as an early warning system to cells in the underlying mucosa, indicating to the host that the mucosal barrier has been penetrated. From this position intestinal epithelial cells might play a crucial role as an outpost of the immune system located in the underlying intestinal mucosa and soluble mediators produced by these cells might function as an early signal to neighbouring

immune cells and are involved in the recruitment of cells during the inflammatory response. Intestinal epithelium is, however, more than a physical barrier and the front of the immune system. An emerging role for the constitutive secretion of cytokines by colonic epithelial cells is to support the growth and development of non epithelial cells in the microenvironment, in particular, lymphocytes (Stadnyk, 1994). It is involved in the maintenance of homeostasis secreting effector molecules, as NO, which for example serves to maintain the colonic mucosal vascular perfusion (Boughton-Smith, 1994). Finally, it participates in repair and healing mechanisms producing chemokines that are necessary for the repair of tissue damage (Miller & Krangel, 1992).

The elucidation of immunoinflammatory process in colonic epithelium combining basic research and pharmacokinetic studies in patients could offer the best approach in the development of new diagnostic test and potential new therapeutic strategies for the effective treatment of IBD. Interrupting or inhibiting the secondary amplification of inflammatory response with selective interventions that target the intestinal cytokine network or other mediators of inflammation will prove of value in the manipulation of intestinal inflammation.

5. CONCLUSIONS

In this study human colonic epithelial cells were found to produce important inflammatory mediators, such as nitric oxide and members of the recently identified chemokine superfamily. These findings provide support for the suggestion that the colonic epithelial cells must be considered as a potentially important source of soluble mediators in intestinal inflammation. Several conclusions can be drawn from this study. Particularly:

1. Colonic epithelial cells have functional receptors for pro-inflammatory and T cell-derived cytokines, which are coupled to a regulation of inflammatory mediators generation, probably contributing to the perpetuation and/or the initiation of the intestinal inflammation.
2. The large amounts of NO that produced from stimulated colonic epithelial cells and the iNOS immunostaining of the epithelial cells in colonic biopsies suggest that these cells but not mononuclear and polymorphonuclear leukocytes may be the major source of NO production in UC. In addition, the basal constitutive production of NO by unstimulated colonic epithelial cells indicates that these cells might participate in the homeostatic role of NO in the intestinal physiology.
3. The production of chemokines by intestinal epithelial cells might be important in the inflammatory process. In this study colonic epithelial cells did not express chemokines unless challenged with cytokines and in addition IL-8 was found to be expressed in inflamed but not normal colonic mucosa. This capacity of colonic epithelial cells to secrete chemokines in response to inflammatory stimuli may be important in the intra-epithelial and lamina propria infiltration by neutrophils and lymphocytes during the intestinal inflammation and indicates a communication between the colonic cells and the immune cells located in the underlying intestinal mucosa.
4. The inhibitory effect of IL-13 and IL-4, on chemokine and nitric oxide production by colonic epithelial cells proposes an anti-inflammatory role of T cell derived cytokines

in the intestinal inflammation. Interestingly, the differential effect of IL-13 and IL-4 on chemokine expression by HT-29 cells suggests that, when considering cytokine network, T cell derived anti-inflammatory cytokines may have a bi-functional role, depending upon the stimulus and the cellular source of chemokines. This dual effect of IL-13 and IL-4 on chemokine expression by colonic epithelial cells may play a crucial role in different stages of the inflammatory process in IBD and contribute to the continuation or the remission of inflammation.

Accepting the above data there is a strong evidence that colonic epithelial cells may play an active role in the inflammatory response, via the generation of inflammatory mediators and T-cell derived cytokines may modulate this process and have a crucial role in the pathogenesis of IBD. These observations suggest a further comprehensive study in order to characterise the spectrum of chemokine and chemokine receptor expression in normal and IBD colonic mucosa and to determine the modulatory effect of T cell derived cytokines and the mechanistic pathway of this modulation of chemokine expression on colonic epithelium.

In addition, we have shown that human colonic epithelial cells express iNOS activity during the intestinal inflammation, which is differently regulated by combinations of cytokines. If these results can be extrapolated *in vivo* then the hypothesis that NO and its metabolites are derived from epithelial cells may be of central pathogenic significance in intestinal inflammation. These results propose further search for naturally occurring and pharmacological agents that might inhibit the production of active nitrogen species and prove of value in the treatment of IBD. Whether the inhibition of NO production will be of therapeutic value in intestinal inflammation awaits further investigation of NO synthase isoforms, and probably the development of selective NOS inhibitors in order to inhibit the excessive NO production by the inducible NOS, without changing the physiological control by the constitutive enzyme.

6. PUBLISHED WORK

PAPERS:

KOLIOS, G., BROWN, Z., ROBSON, R.L., ROBERTSON, D.A.F., AND WESTWICK, J. (1995) Inducible nitric oxide synthase activity and expression in a human colonic epithelial cell line, HT-29. *Br. J. Pharmacol.* **116**, 2866-2872.

KOLIOS, G., ROBERTSON, D.A.F., JORDAN, N.J., MINTY, A., CAPUT, D., FERRARA, P., AND WESTWICK, J. (1996) Interleukin-8 production by the human colon epithelial cell line HT-29: Modulation by interleukin 13. *Br. J. Pharmacol.* **119**, 351-359.

KOLIOS, G., ROONEY, N., MURPHY, C.T., ROBERTSON, D.A.F., AND WESTWICK, J. (1997) Expression of inducible nitric oxide (iNOS) activity in human colon epithelial cells: Modulation by T-lymphocyte derived cytokines. *Gut* **in press.**

ABSTRACTS:

KOLIOS, G., ROBERTSON, D.A.F., AND WESTWICK, J. (1994) Interleukin-13 enhances IL-1a but not TNF-α-induced chemokine expression in human colonic epithelial cells. *Gut* **35**, S47

KOLIOS, G., ROBERTSON, D.A.F., AND WESTWICK, J. (1995) Pro-inflammatory cytokine-induced nitric oxide production and iNOS mRNA expression in human colonic epithelial cells. *Gastroenterology* **108**, A851

KOLIOS, G., ROBSON, R.L., BROWN, Z., ROBERTSON, D.A.F., AND WESTWICK, J. (1995) Modulation of the inducible nitric oxide synthase (iNOS) in the human colonic epithelial cell line HT-29. *Br. J. Pharmacol.* **114**, 133P

KOLIOS, G., MURPHY, C.T., ROBERTSON, D.A.F., AND WESTWICK, J. (1996) Modulation of inducible nitric oxide (iNOS) activity in the human colon epithelial cell line HT-29: Dual effect of interleukin-13 (IL-13). *Br. J. Pharmacol.* **117**, 87P

KOLIOS, G., ROBERTSON, D.A.F., ROONEY, N., AND WESTWICK, J. (1996) Inducible nitric oxide synthase (iNOS) activity in ulcerative colitis: suppression by the anti-inflammatory cytokine IL-13. *Gut* **38**, A637

KOLIOS, G., ROBERTSON, D.A.F., AND WESTWICK, J. (1996) Interleukin-13 regulates the nitric oxide production in human colonic epithelial cells. Comparison with interleukin-4 and interleukin-10. *Gastroenterology* **110**, A940

KOLIOS, G., JORDAN, N.J., LEITHEAD, J., ROBERTSON, D.A.F., AND WESTWICK, J. (1997) Differential effect of IL-13 on C-X-C and C-C chemokine expression by colonic epithelial cells. *Gastroenterology* **112**, A1017

KOLIOS, G., WRIGHT, K.L., LINEHAN, J.D., ROBERTSON, D.A.F., AND WESTWICK, J. (1997) Regulation of nitric oxide production in human colonic mucosa. Gastroenterology **112**, A1018

7. REFERENCES

ABREUMARTIN, MT & TARGAN, SR (1996) Regulation of immune responses of the intestinal mucosa. *Crit. Rev. Immunol.* **16**, 277-309.

ADAMS, DH & SHAW, S (1994) Leucocyte-endothelial interactions and regulation of leucocyte migration. *Lancet* **343**, 831-836.

AHRENSTEDT, O, KNUTSON, L, NILSSON, B, NILSON-EKDAHL, K, ODIND, B & HALLGREN, R (1990) Enhanced local production of complement components in the small intestines of patients with Crohn's disease. *N. Engl. J. Med.* **322**, 1345-1349.

AHRENSTEDT, O, HALLGREN, R & KNUTSON, L (1994) Jejunal release of prostaglandin-E(2) in Crohn's disease; relation to disease activity and first degree relatives. *J. Gastroenterol. Hepatol.* **9**, 539-543.

ALICAN, I & KUBES, P (1996) A critical role for nitric oxide in intestinal barrier function and disfunction. *Am. J. Physiol.* **33**, G225-G237.

ALLGAYER, H, RANG, S, KLOTZ, U, BOHNE, P, RETEY, J, KRUIS, W & GUGLER, R (1994) Superoxide inhibition following different stimuli of respiratory burst and metabolism of aminosalicylates in neutrophils. *Dig. Dis. Sci.* **39**, 145-151.

ALMER, S, KALD, B, FRANZEN, L, STROM, M & TAGESSON, C (1996) Concentrations of platelet-activating factor in rectal mucosa in patients with ulcerative colitis. *Eur. J. Surg.* **5**, 391-396.

ANHFELT-RONNE, I, NIELSEN, OH, CHRISTENSEN, A, LANGHOLZ, E, BINDER, V & RIIS, P (1990) Clinical evidence supporting the radical scavenger mechanism of 5-aminosalisylic acid. *Gastroenterology* **98**, 1162-1169.

ANTHONY, D, SAVAGE, F, HEMBRY, R & BOULOS, P (1994) Protease expression in experimental colitis. *Agents & Actions* **41**, C201-C203.

ASAKURA, H, TSUCHIYA, M, ALSO, S, WATANABE, M, KOBAYASHI, K, HIBI, T, ANDO, K, TAKATA, H & SEKIGUCHI, S (1982) Association of the human lymphocyte-DR2 antigen with Japanese ulcerative colitis. *Gastroenterology* **82**, 413-418.

ASANO, K, CHEE, CBE, GASTON, B, LILLY, CM, GERARD, C, DRAZEN, JM & STAMLER, JS (1994) Constitutive and inducible nitric oxide synthase gene expression, regulation and activity in human lung epithelial cells. *Proc. Natl. Acad. Sci. USA* **91**, 10089-10093.

BAGGIOLINI, M, WALZ, A & KUNKEL, SL (1989) Neutrophil-activating peptide-1/interleukin 8, a novel cytokine that activates neutrophils. *J. Clin. Invest.* **84**, 1045-1049.

BAGGIOLINI, M, DEWALD, B & MOSER, B (1994) Interleukin-8 and related chemotactic cytokines--CXC and CC chemokines. *Adv. Immunol.* **55**, 97-179.

BARCLAY, GR, MCKENZIE, H, PENNINGTON, J, PARRATT, D & PENNINGTON, CR (1992) The effect of dietary yeast on the activity of stable chronic Crohn's disease. *Scand. J. Gastroenterol.* **27**, 196-200.

BARKER, JNWN, JONES, ML, MITRA, RS, CROCKETT-TORABE, E, FANTONE, JC, KUNKEL, SL, WARREN, JS, DIXIT, VM & NICKOLOFF, BJ (1991) Modulation of keratinocyte-derived interleukin-8, which is chemotactic for neutrophils and T lymphocytes. *Am. J. Pathol.* **139**, 869-876.

BARRETT, SML, STANDEN, PJ, LEE, AS, HAWKEY, CJ & LOGAN, RFA (1996) Personality, smoking and inflammatory bowel disease. *Eur. J. Gastroenterol. Hepatol.* **8**, 651-655.

BAUMEISTER, B, SCHMIDT, C, HELISCH, A & KIPNOWSKI, J (1996) Increased prostagladin E(2) and leukotriene B(4) synthesis in isolated colonic mucosal cells in inflammatory bowel disease. A preliminary report. *J. Clin. Gastroenterol.* **22**, 117-120.

BAYLESS, TM (1995) Gut-brain interactions in inflammatory bowel disease. A clinician perspective. *Can. J. Gastroenterol.* **9**, 273-276.

BAZAN, JF, BACON, KB, HARDIMAN, G, WANG, W, SOO, K, ROSSI, D, GREAVES, DR, ZLOTNIK, A & SCHALL, TJ (1997) A new class of membrane-bound chemokine with a CX_3C motif. *Nature* **385**, 640-644.

BECKMAN, JS, BECKMAN, TW, CHEN, J, MARSHALL, PA & FREEMAN, BA (1990) Apparent hydroxyl radical production by peroxinitrite; Implication for endothelial injury from nitric oxide and superoxide. *Proc. Natl. Acad. Sci. USA* **87**, 1620-1624.

BERK, T, GORDON, SJ, CHOI, HY & COOPER, HS (1985) Cytomegalovirus infections of the colon: a possible role in exacerbations of inflammatory bowel disease. *Am. J. Gastroenterol.* **80**, 355-360.

BERN, MJ, STURBAUM, CN, KARAYALCIN, SS, BERSCHNEIDER, HM, WACHSMAN, JT & POWELL, DW (1989) Immune system control of rat and rabbit colonic electrolyte transport. *J. Clin. Invest.* **83**, 1810-1820.

BERTRAN, X, MANE, J, FERNADEZ-BANARES, F, CASTELLA, A, BARTOLI, R, OJANGUREN, I & GASSULL, MA (1996) Intracolonic adminisration of Zileuton, a selective 5-lipoxygenase inhibitor, accelerates healing in a rat model of chronic colitis. *Gut* **38**, 899-904.

BEUTLER, B & CERAMI, A (1995) Cachectin and tumour necrosis factor as two sides of the same biological coin. *Nature* **320**, 584-588.

BIOQUE, G, CRUSIUS, JBA, KOUTROUBAKIS, I, BOUMA, G, KOSTENSE, PJ, MEUWISSEN, SGM & PENA, AS (1995) Allelic polymorphism in IL-1beta and IL-receptor antagonist (IL-1RA) genes in inflammatory bowel disease. *Clin. Exp. Immunol.* **102**, 379-383.

BIOQUE, G, BOUMA, G, CRUCIUS, JBA, KOUTROUBAKIS, I, KOSTENSE, PJ, MEUWISSEN, SGM & PENA, AS (1996) Evidence for genetic heterogeneity in IBD. The interleukin-2 receptor antagonist in the predisposition to suffer from ulcerative colitis. *Eur. J. Gastroenterol. Hepatol.* **8**, 105-110.

BJARNASON, I, MACPHERSON, A & HOLLANDER, D (1995) Intestinal permeability: an overview. *Gastroenterology* **108**, 1566-1581.

BJARNASON, I & PETERS, TJ (1987) Helping the mucosa make sense of macromolecules. *Gut* **28**, 1057-1061.

BJORCK, S, DAHLSTROM, A & AHLMAN, H (1989) Topical treatment of ulcerative proctitis with lidocaine. *Scand. J. Gastroenterol.* **24**, 1061-1072.

BOEHM, U, KLAMP, T, GROOT, M & HOWARD, JC (1997) Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* **15**, 749-795.

BOLTON, RP, SHERIFF, RI & READ, AE (1980) Clostiridium difficile associated diarrhoea: a role in inflammatory bowel disease? *Lancet* **1**, 383-384.

BOUGHTON-SMITH, NK, EVANS, SM, HAWKEY, CJ, COLE, AT, BALSITIS, M, WHITTLE, BJR & MONCADA, S (1993) Nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Lancet* **342**, 338-340.

BOUGHTON-SMITH, NK (1994) Pathological and therapeutic implications for nitric oxide in inflammatory bowel disease. *J. Royal Soc. Medicine* **87**, 312-313.

BOYKO, EJ, THEIS, MK, VAUGHAN, TL & NICOL-BLADES, B (1994) Increased risk of inflammatory bowel disease associated with oral contraceptive use. *Am. J. Epidemiol.* **140**, 268-278.

BRADFORD, MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248-254.

BRAEGGER, CP, NICHOLLS, S, MURCH, SH, STEPHENS, S & MACDONALD, TT (1992) Tumour necrosis factor alpha in stool as a marker of intestinal inflammation. *Lancet* **339**, 89-91.

BRANDTZAEG, P (1973) Structure synthesis and transfer for mucosal immunoglobulins. *Annu. Rev. Immunol.* **1246**, 417-429.

BREDT, DS, HWANG, PM, GLATT, CE, LOWENSTEIN, C, REED, RR & SNYDER, SH (1991) Cloned and expressed nitric oxide synthase structurally resembles cytochrome P-450 reductase. *Nature* **351**, 714-718.

BREUER, RI, BUTO, SK, CHRIST, ML, BEAN, J, VERNIA, P, PAOLUZI, P, DIPAOLO, MC & CAPRILLI, R (1991) Rectal irrigation with short chain fatty acids for distal ulcerative colitis. Preliminary report. *Dig. Dis. Sci.* **36**, 185-187.

BREUER, RI, SOERGEL, KH, LASHNER, BA, CHRIST, ML, HANAUER, SB, VANAGUNAS, A, HARIG, JM, KESHAVARZIAN, A, ROBINSON, M, SELLIN, JH, WEINBERG, D, VIDICAN, DE, FLEMAL, KL & RADEMAKER, AW (1997) Short chain fatty acid rectal irrigation for left-sided ulcerative colitis: A randomised, placebo controlled trial. *Gut* **40**, 485-491.

BREUERKATSCHINKSKI, BD, HOLLANDER, N & GOEBELL, H (1996) Effect of cigarette smoking on the course of Crohn's disease. *Eur. J. Gastroenterol. Hepatol.* **8**, 225-228.

BRIAN, JE, FARACI, FM & HEISTAD, DD (1996) Recent insights into the regulation of cerebral circulation. *Clin. Exp. Pharmacol. Physiol.* **23**, 449-457.

BROWN, JF, TEPPERMAN, BL, HANSON, PJ, WHITTLE, BJR & MONCADA, S (1992) Differential distribution of nitric oxide synthase between cell fractions isolated from the rat gastric mucosa. *Biochem. Biophys. Res. Commun.* **184**, 680-685.

BROWN, Z, STRIETER, RM, CHENSUE, SW, CESKA, M, LINDLEY, I, NEILD, GH, KUNKEL, SL & WESTWICK, J (1991) Cytokine-activated human mesangial cells generate the neutrophil chemoattractant, interleukin 8. *Kidney Int.* **40**, 86-90.

BROWN, Z, GERRITSEN, ME, CARLEY, WW, STRIETER, RM, KUNKEL, SL & WESTWICK, J (1994) Chemokine gene expression and secretion by cytokine-activated human microvascular endothelial cells: Differential regulation of monocyte chemoattractant protein-1 and interleukin 8 in response to interferon-gamma. *Am. J. Pathol.* **145**, 913-921.

BRYNSKOV, J, NIELSEN, OH, AHNFELT-RONNE, I & BENDTZEN, K (1992) Cytokines in inflammatory bowel disease. *Scand. J. Gastroenterol.* **27**, 897-906.

BURLEIGH, DE (1992) N^G-nitro-L-arginine reduces nonadrenergic, noncholinergic relaxations of human gut. *Gastroenterology* **102**, 679-683.

CALLARD, RE, MATTHEWS, DJ & HIBBERT, L (1996) IL-4 and IL-13 receptors: are they one and the same? *Immunol. Today* **17**, 108-110.

CAMPBELL, JM, FAHEY, GC, JR., LICHTENSTEIGER, CA, DEMICHELE, SJ & GARLEB, KA (1997) An enteral formula containing fish oil, indigestible oligosaccharides, gum arabic and antioxidants affects plasma and colonic phospholipid fatty acid and prostaglandin profiles in pigs. *J. Nutr.* **127**, 137-145.

CARLOS, TM & HARLAN, JM (1994) Leukocyte-endothelial adhesion molecules. *Blood* **84**, 2068-2101.

CARR, MW, ROTH, SJ, LUTHER, E, ROSE, SS & SPRINGER, TA (1994) Monocyte chemoattractant protein 1 acts as a T-lymphocyte chemoattractant. *Proc. Natl. Acad. Sci. USA* **91**, 3652-3656.

CASELLAS, F, PAPO, M, GUARNER, F, ANTOLIN, M, SEGURA, RM, ARMENGOL, JR & MALAGELADA, JR (1995) Effects of thromboxane synthase inhibition on in vivo release of inflammatory mediators in chronic ulcerative colitis. *Eur. J. Gastroenterol. Hepatol.* **7**, 221-226.

CASELLAS, F & GUARNER, F (1996) Eicosanoids in inflammatory bowel disease. Therapeutic implications. *Clin. Immunother.* **6**, 333-340.

CASOLA, A, ESTES, MK, CRAWFORD, SE, ERNST, PB, GAROFALO, R, OGRA, P & CROWE, SE (1997) Rotavirus infection of intestinal epithelial cells induces chemokine secretion. *Gastroenterology* **112**, A944

CATTELL, V & COOK, HT (1993) Nitric oxide; Role in the physiology and pathology of the glomerulus. *Exp. Nephrol.* **5**, 265-280.

CENCI, E, ROMANI, L, MENCACCI, A, SPACCAPELO, R, SCHIAFFELLA, E, PUCCETTI, P & BISTONI, F (1993) Interleukin-4 and interleukin-10 inhibit nitric oxide-dependent macrophage killing of candida-albicans. *Eur. J. Immunol.* **23**, 1034-1038.

CERF-BENSUSSAN, N, QUARONI, A, KURNICK, JT & BHAN, AK (1984) Intraepithelial lymphocytes modulate Ia expression by intestinal epithelial cells. *J. Immunol.* **132**, 2244-2252.

CERF-BENUSSAN, N & GAY-GRAND, D (1991) Intestinal epithelial lymphocytes. *Gastroenterol.Clin.North Am.* **20**, 549-574.

CESKA, M., EFFENBERGER, F., PEICHI, P., AND PURSCH, E. (1989) Purification and characterisation of monoclonal andpolyclonal antibodies to neutrophil activation peptide (NAP-1). The developement of highly sensitive ELISA methods for the determination of NAP-1 and anti-NAP-1 antibodies. *Cytokine* **1**, 136-141.

CHANTRET, I, BARBAT, A, DUSSAULX, E, BRATTAIN, MG & ZWEIBAUM, A (1988) Epithelial polarity, villin expression, and enterocytic differentiation of cultured human colon carcinoma cells: A survey of twenty cell lines. *Cancer Res.* **48**, 1936-1942.

CHARLES, IG, PALMER, RMJ, HICKERY, MS, BAYLISS, MT, CHUBB, AP, HALL, VS, MOSS, DW & MONCADA, S (1993) Cloning, characterization, and expression of a cDNA encoding an inducible nitric oxide synthase from human chondrocyte. *Biochemistry* **90**, 11419-11423.

CHARO, IF, MYERS, SJ, HERMAN, A, FRANCI, C, CONNOLLY, AJ & COUGHLIN, SR (1994) Molecular cloning and functional expression of two monocyte chemoattractant protein 1 receptors reveals alternative splicing of the carboxyl - terminal tails. *Proc. Natl. Acad. Sci. USA* **91**, 2752-2756.

CHIBA, M, IIZUKA, M, HORIE, Y, ISHII, N & MASAMUNE, O (1994) Expression of HLA-DR antigens on colonic epithelium around lymph follicles. *Dig. Dis. Sci.* **39**, 1918-1923.

CICLITIRA, PJ (1993) Does Crohn's disease have a mycobacterial basis? *Br. Med. J.* **306**, 733-734.

CLAUSEN, MR & MORTENSEN, PB (1995) Kinetic studies on colonocyte metabolism of short chain fattyacids and glucose in ulcerative colitis. *Gut* **37**, 684-689.

COHEN, RD & HANAUER, SB (1996) Nicotine in ulcerative colitis; How does it work and how can we use it? *Clin. Immunother.* **5**, 169-174.

COLE, AT, FILIPOWICZ, B, HYMANTAYLOR, P & HAWKEY, CJ (1994) Low dose aspirin selective inhibition of rectal dialysis thromboxane B2 in healthy volunteers. *Aliment. Pharmacol. Therap.* **8**, 521-526.

COLE, AT, PILKINGTON, BJ, MCLAUGHLAN, J, SMITH, C, BALSITIS, M & HAWKEY, CJ (1996) Mucosal factors inducing neutrophil movement in ulcerative colitis. The role of interleukin 8 and leukotriene B4. *Gut* **39**, 248-254.

COLLINS, CE, CAHILL, M, NEWLAND, A & RAMPTON, DS (1994) Platelets circulate in an activated state in inflammatory bowel disease. *Gastroenterology* **106**, 840-845.

COLLINS, CE, BENSON, MJ, BURNHAM, WR & RAMPTON, DS (1996) Picotamide inhibition of excess in vitro thromboxane B2 release by colorectal mucosa in inflammatory bowel disease. *Aliment. Pharmacol. Therap.* **10**, 315-320.

COLOTTA, F, RE, F, MUZIO, M, POLENTARUTTI, N, MINTY, A, CAPUT, D, FERRARA, P & MANTOVANI, A (1994) Interleukin-13 induces expression and release of interleukin-1 decoy receptor in human polymorphonuclear cells. *J. Biol. Chem.* **269**, 12403-12406.

COOK, TH, BUNE, AJ, JANSEN, AS, TAYLOR, GM, LOI, RK & CATTELL, V (1994) Cellular localization of inducible nitric oxide synthase in experimental endotoxic shock in the rats. *Clin. Sci.* **87**, 179-186.

COPE, GF & HEATLEY, RV (1992) Cigarette smoking and intestinal defences. *Gut* **33**, 721-723.

COSENTINO, F & LUSCHER, TF (1995) Maintenance of vascular integrity: role of nitric oxide and other bradykinin madiators. *Eur. Heart J.* **16 SK**, 4-12.

CROITORU, K & ERNEST, PB (1992) Leukocyes in the intestinal epithelium. An unusual immunological compartment revisited. *Regional Immunol* **4**, 63-69.

CUMMINGS, JH & MACFARLANE, GT (1991) The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.* **70**, 443-459.

CUNHA, FQ, MONCADA, S & LIEW, FY (1992) Interleukin-10 (IL-10) inhibits the induction of nitric oxide synthase by interferon gamma in murine macrophages. *Biochem. Biophys. Res. Commun.* **182**, 1155-1159.

DAIG, R, ANDUS, T, ASCHENBRENNER, E, FALK, W, SCHÖLMERICH, J & GROSS, V (1996) Increased interleukin 8 expression in the colon mucosa of patients with inflammatory bowel disease. *Gut* **38**, 216-222.

DAUGHERTY, BL, SICILIANO, SL, DEMARTINO, JA, MALKOWITZ, L, SIROTINA, A & SPRINGER, MS (1996) Cloning, expression and characterization of the human eosinophil eotoxin receptor. *J. Exp. Med.* **183**, 2349-2354.

DAVIES, NM (1995) Toxicity of non-steroidal anti-inflammatory drugs in the large intestine. *Dis. Colon Rectum* **38**, 1311-1321.

DE WAAL MALEFYT, R, ABRAMS, J, BENNET, B, FIGDOR, CG & DE VRIES, JE (1991) Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes - an autoregulatory role for IL-10 produced by monocytes. *J. Exp. Med.* **174**, 1208-1220.

DE WAAL MALEFYT, R, FIGDOR, CG, HUIJBENS, R, MOHAN-PETERSON, S, BENNETT, B, CULPEPPER, J, DANG, W, ZURAWSKI, G & DE VRIES, JE (1993) Effects of IL-13 on phenotype, cytokine production, and cytotoxic function of human monocytes: Comparison with IL-4 and modulation by IFN-gamma or IL-10. *J. Immunol.* **151**, 6370-6381.

DE WAAL MALFYT, R, ABRAMS, J, BENNETT, B, FIGDOR, CG & DE VRIES, JE (1991) Interleukin 10 (IL-10) inhibits cyutokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *J. Exp. Med.* **174**, 1209-1220.

DEFRANCE, T, CARAYON, P, BILLIAN, G, GUILLEMOT, J-C, MINTY, A, CAPUT, D & FERRARA, P (1994) Interleukin 13 is a B cell stimulating factor. *J. Exp. Med.* **179**, 135-143.

DELACADENA, RA, LASKIN, KJ, PIXLEY, RA, SARTOR, RB, SCHWAB, JH, BACK, N, BEDI, GS, FISHER, RS & COLMAN, RW (1991) Role of kallikrein-kinin system in pathogenesis of bacterial cell wall-induced inflammation. *Am. J. Physiol.* **260**, G213-G219.

DEROCQ, J-M, SEGUI, M, POINOT-CHAZEL, C, MINTY, A, CAPUT, D, FERRARA, P & CASELLAS, P (1994) Interleukin-13 stimulates interleukin-6 production by human keratinocytes: Similarity with interleukin-4. *FEBS Letts.* **343**, 32-36.

DIAS, VC, WALLACE, JI & PARSONS, HG (1992) Modulation of cellular phospholipid fatty acid leukotriene B4 synthesis im the human intestinal cell (CaCo-2). *Gut* **33**, 622-627.

DIELEMAN, LA, ELSON, CO, TENNYSON, GS & BEAGLEY, KW (1996) Kinetics of cytokine expression during healing of acute colitis in mice. *Am. J. Physiol.* **34**, G130-G136.

DINARELLO, CA (1996) Biologic basis for interleukin-1 in disease. *Blood* 2095-22147.

DING, A, NATHAN, CF, GRAYCAR, J, DERYNK, R, STUEHR, DJ & SRIMAL, S (1990) Macrophage deactivating factor and transforming growth factor beta-1, factor beta-2, and factor beta-3 inhibit induction of macrophage nitrogen oxide synthesis by IFN-gamma. *J. Immunol.* **145**, 940-944.

DOHERTY, TM, KASTELEIN, R, MENON, S, ANDRADE, S & COFFMAN, RL (1993) Modulation of murine macrophage function by IL-13. *J. Immunol.* **151**, 7151-7160.

DONLON, TA, KRENSKY, AM, WALLACE, MR, COLLINS, FS, LOVETT, M & CLAYBERGER, C (1990) Localization of a human T-cell specific gene,RANTES (D17S136E),to chromosome 17q11.2-q12. *Genomics* **6**, 548-553.

DOYLE, AG, HERBEIN, G, MONTANER, LJ, MINTY, AJ, CAPUT, D, FERRARA, P & GORDON, S (1994) Interleukin-13 alters the activation state of murine macrophages *in*

vitro: Comparison with interleukin-4 and interferon-gamma. *Eur. J. Immunol.* **24**, 1441-1445.

DRAGIC, T, LITWIN, V, ALLAWAY, GP, MARTIN, SR, HUANG, YX, NAGASHIMA, KA, CAYANAN, C, MADDON, PJ, KOUP, RA, MOORE, JP & PAXTON, WA (1996) HIV-1 entry into $CD4^+$ cells is mediated by the chemokine receptor CC-CKR-5. *Nature* **381**, 667-673.

DRAPIER, JC & HIBBS, JB (1986) Murine cytotoxic activated macrophages inhibit aconitase in tumour cells. Inhibition involves the iron-sulfur prosthetic group and is reversible. *J. Clin. Invest.* **78**, 790-797.

DUCHMANN, R, SCHMITT, E, KNOLLE, P, ZUMBUSCHENFELDE, KHM & NEURATH, M (1996) Tolerance towards resident intestinal flora in mice is abrogated in experimental colitis and restored by treatment with interleukin-10 or antibodies to interleukin-12. *Eur. J. Immunol.* **26**, 934-938.

DUMONCEAU, JM, VANGOSSUM, A, ADLER, M, FONTEYNE, PA, VANVOOREN, JP, DEVIERE, J & PORTAELS, F (1996) No Mycobacterium paratuberculosis found in Crohn's disease using the polymerase chain reaction. *Dig. Dis. Sci.* **41**, 421-426.

EBERT, EC, WRIGHT, SH, LIPSHUTZ, WH & HAUPTMAN, SP (1984) T-cell abnormalities in inflammatory bowel disease are mediated by interleukin-2. *Clin. Immunol. Immunopathol.* **33**, 232-244.

EBERT, EC (1995) Human intestinal intraepithelial lymphocytes have potent chemotactic activity. *Gastroenterology* **109**, 1154-1159.

ECKMANN, L, JUNG, HC, SCHARER-MALY, C, PANJA, A, MORZYCKA-WROBLEWSKA, E & KAGNOFF, MF (1993) Differential cytokine expression by human intestinal epithelial cell lines: Regulated expression of interleukin 8. *Gastroenterology* **105**, 1689-1697.

EHRHARDT, RO, LUDVIKSSON, BR, GRAY, B, NEURATH, M & STROBER, W (1997) Induction and prevention of colonic inflammation in IL-2 deficient mice. *J. Immunol.* **158**, 566-573.

EKBOM, A, WAKEFIELD, AJ, ZACK, M & ADAMI, HO (1994) Perinatal measles infection and subsequent Crohn's disease. *Lancet* **344**, 508-510.

EKBOM, A, DASZAK, P, KRAAZ, W & WAKEFIELD, AJ (1996) Crohn's disease after in utero measles virus exposure. *Lancet* **348**, 515-517.

ELLIS, A (1995) Genetics in Gastroenterology. In *Gastroenterology*. 4th Ed. Ed M. Bocus. pp. 3287-3300.

EMERIT, J, PELLETIER, S, TOSONI-VERLINGUE, D & MOLLET, M (1989) Phase II trial of cooper zinc superoxide dismutase in treatment of Crohn's disease. *Free Radic. Biol. Med.* **7**, 145-149.

FAIS, S, CAPOBIANCHI, MR & PALLONE, F (1991) Spontaneous release of interferon-gamma by intestinal lamina propria lymphocytes in Crohn's disease. Kinetics of in vivo response to interferon g inducers. *Gut* **32**, 403-407.

FELSEN, J (1936) The relationship of bacillary dysentery to distal ileitis, chronic ulcerative colitis and non specific intestinal granuloma. *Ann. Internal Med.* **10**, 645-669.

FENG, N, SCHNYDER, B, VONDERSCHMITT, DJ, RYFFEL, B & LUTZ, RA (1995) Characterization of interleukin-13 receptor in carcinoma cell lines and human blood cells and comparison with the interleukin-4 receptor. *J. Rec. Sign. Transd. Res.* **15**, 931-949.

FENG, Y, BRODER, CC, KENNEDY, PE & BERGER, EA (1996) HIV-1 entry co-factor; functional cDNA cloning of a 7-transmembrane, G-protein coupled receptor. *Science* **272**, 872-877.

FERRARIS, L, KARMELI, F, ELIAKIM, R, KLEIN, J, FIOCCHI, C & RACHMILEWITZ, D (1993) Intestinal epithelial cells contribute to the advanced generation of platelet activating factor in ulcerative colitis. *Gut* **34**, 665-668.

FINNIE, IA, DWARAKANATH, AD, TAYLOR, BA & RHODES, JM (1995) Colonic mucin synthesis is increased by sodium butyrate. *Gut* **36**, 93-99.

FINNIE, IA, CAMPBELL, BJ, TAYLOR, BA, MILTON, JD, SADEK, SK, YU, LG & RHODES, JM (1996) Stimulation of colonic mucin synthesis by corticosteroids and nicotine. *Clin. Sci.* **91**, 359-364.

FIOCCHI, C (1989) Lymphokines and the intestinal immune response. Role in inflammatory bowel disease. *Immunol. Invest.* **18**, 91-102.

FIOCCHI, C, BINION, DG & KATZ, JA (1994) Cytokine production in the human gastrointestinal tract during inflammation. *Curr. Opin. Gastroenterol.* **2**, 639-644.

FIORENTINO, DF, BOND, MW & MOSMANN, TR (1989) Two types of mouse helper T cell. IV Th2 clones secrete a factor that inhibits cytokine production by Th1 clones. *J. Exp. Med.* **170**, 2081-2095.

FIORENTINO, DF, ZLOTNIK, A, MOSMANN, TR, HOWARD, M & O'GARRA, A (1991) IL-10 inhibits cytokine production by activated macrophages. *J. Immunol.* **147**, 3815-3822.

FRANCI, C, WONG, NS, VAN DAMME, J, PROOST, P & CHARO, IF (1995) Monocyte chemoattractant protein-3, but not monocyte chemoattractant protein-2, is a functional ligand of the human monocyte chemoattractant protein-1 receptor. *J. Immunol.* **154**, 6511-6517.

FRIEDLAND, JS, REMICK, DG, SHATTOCK, R & GRIFFIN, GE (1992) Secretion of interleukin-8 following phagocytosis of Mycobacterium tuberculosis by human monocyte cell line. *Eur. J. Immunol.* **22**, 1373-1378.

FUKUTO, JM & CHAUDHURI, G (1995) Inhibition of constitutive and inducible nitric oxide synthase. Potential selective inhibition. *Annu. Rev. Pharmacol. Toxicol.* **35**, 165-194.

GARCIAZEPEDA, EA, ROTHENBERG, ME, OWNBEY, RT, CELESTIN, J, LEDER, P & LUSTER, AD (1996) Human eotaxin is a specific chemoattractant for eosinophil cells and provides a new mechanism to explain tissue eosinophilia. *Nature Med.* **2**, 449-456.

GELLER, DA, LOWENSTEIN, CJ, SHAPIRO, RA, NUSSLER, AK, DI SILVIO, M, WANG, SC, NAKAYAMA, DK, SIMMONS, RL, SNYDER, SH & BILLIAR, TR (1993) Molecular cloning and expression of inducible nitric oxide synthase from human hepatocytes. *Proc. Natl. Acad. Sci. USA* **90**, 3491-3495.

GIBSON, P & ROSELLA, O (1995) Interleukin 8 secretion by colonic crypt cells in vitro: response to injury suppressed by butyrate and enhanced in inflammatory bowel disease. *Gut* **37**, 536-543.

GILAT, D, HERSHKOVIZ, R, MEKORI, YA, VLODAVSKY, I & LIDER, O (1994) Regulation of adhesion of CD4+ T Lymphocytes to intact or heparinase-treated subendothelial extracellular matrix by diffusible or anchored RANTES and MIP-1b. *J. Immunol.* **153**, 4899-4906.

GILLITZER, R, BERGER, R, MIELKE, V, MULLER, C, WOLFF, K & STINGL, G (1991) Upper keratinocytes of psoriatic skin lesions express high levels of NAP/IL-8 mRNA in situ. *J. Invest. Dermatol.* **97**, 73-79.

GODKIN, AJ, DEBELDER, AJ, VILLA, L, WONG, A, BEESLEY, JE, KANE, SP & MARTIN, JF (1996) Expression of nitric oxide synthase in ulcerative colitis. *Eur. J. Clin. Invest.* **26**, 867-872.

GOLDMAN, M, VELU, T & PRETOLANI, M (1997) Interleukin-10: Actions and therapeutic potential. *BioDrugs* **7**, 6-14.

GREEN, JT, THOMAS, GAO, RHODES, J, EVANS, BK, RUSSELL, MAH, FEYERABEND, C, FULLER, GS, NEWCOMBE, RG & SANDBORN, WJ (1997) Pharmacokinetics of nicotine carbomer enemas: A new treatment modality for ulcerative colitis. *Clin. Pharmacol. Ther.* **61**, 340-348.

GREENEWALD, BD & JAMES, SP (1995) Immunology of inflammatory bowel disease. *Curr. Opin. Gastroenterol.* **11**, 298-304.

GREENFIELD, SM, HAMBLIN, AS, SHAKOOR, ZS, TEARE, JP, PUNCHARD, NA & THOMPSON, RPH (1993) Inhibition of leukocyte adhesion molecule upregulation by tumour necrosis factor a: A novel mechanism of action of sulphasalazine. *Gut* **34**, 252-256.

GRIMM, MC, PULLMAN, WE, BENNETT, GM, SULLIVAN, PJ, PAVLI, P & DOE, WF (1995) Direct evidence of monocyte recruitment to inflammatory bowel disease mucosa. *J. Gastroenterol. Hepatol.* **10**, 387-395.

GRIMM, MC, ELSBURY, SKO, PAVLI, P & DOE, WF (1996) Enhanced expression and production of Monocyte Chemoattractant Protein-1 in inflammatory bowel disease mucosa. *J. Leukoc. Biol.* **59**, 804-812.

GRIMM, MC & DOE, WF (1996) Chemokines in inflammatory bowel disease mucosa. Expression of RANTES, Macrophage Inflammatory Protein (MIP)-1-alpha, MIP-1-beta, and Gamma Interferon Inducible Protein-10 by macrophages, lymphocytes, endothelial cells and granulomas. *Inflamm. Bowel Dis.* **2**, 88-96.

GRISHAM, MB, WARE, K, GILLELAND, HE, GILLELAND, LB, ABELL, CL & YAMADA, T (1992) Neutrophil-mediated nitrosamine formation: a role of nitric oxide in rats. *Gastroenterology* **103**, 1260-1266.

GRISHAM, MB (1993) Nitric oxide production by intestinal epithelial cells. *Gastroenterology* **104(4)**, A710

GRISHAM, MB (1994) Oxidants and free radicals in inflammatory bowel disease. *Lancet* **344**, 859-861.

GRISHAM, MB & MILES, AM (1994) Effects of aminosalicylates and immunosuppressive agents on nitric oxide-dependent N-nitrosation reactions. *Biochem. Pharmacol.* **47**, 1897-1902.

GROSS, SS, STUEHR, DJ, AISAKA, K, JAFFE, EA, LEVI, R & GRIFFITH, OW (1990) Macrophage and endothelial cell nitric oxide synthesis: Cell-type selective inhibition by N^G-aminoarginine, N^G-nitroarginine and N^G-methylarginine. *Biochem. Biophys. Res. Commun.* **170**, 96-103.

GROSS, SS, JAFFE, EA, LEVI, R & KILBOURN, RG (1991) Cytokine-activated endothelial cells express an isotype of nitric oxide synthase which is tetrahyrdrobiopterin-dependant, calmodulin-independant and inhibited by arginine analogs with a rank-order of potency characteristic of activated macrophages. *Biochem. Biophys. Res. Commun.* **178**, 823-829.

GROSS, V, ARNDT, H, ANDUS, T, PALITZSCH, KD & SCHOLMERICH, J (1994) Free radicals in inflammatory bowel diseases; pathophysiology and therapeutic implications. *Hepato-Gastroenterol.* **41**, 320-327.

GROSS, V, ANDUS, T, DAIG, R, ASCHENBRENNER, E, SCHÖLMERICH, J & FALK, W (1995) Regulation of interleukin-8 production in a human colon epithelial cell line (HT-29). *Gastroenterology* **108**, 653-661.

GROTTRUPWOLFERS, E, MOELLER, J, KARBACH, U, MULLERLISSNER, S & ENDRES, S (1996) Elevated cell associated levels of interleukin-1 beta and interleukin-6 in inflamed mucosa of inflammatory bowel disease. *Eur. J. Clin. Invest.* **26**, 115-122.

GUIMBAUD, R, IZZO, A, MARTINOLLE, JP, VIDON, N, COUTURIER, D, BENVENISTE, J & CHAUSSADE, S (1995) Intraluminal excretion of PAF, lysoPAF and acetylhydrolase in patients with ulcerative colitis. *Dig. Dis. Sci.* **40**, 2635-2640.

GUO, FH, DERAEVE, HR, RICE, TW, STUEHR, DJ, THUNNISSEN, FB & ERZURUM, SC (1995) Continuous nitric oxide synthasis by inducible nitric oxide synthase in normal human airway epithelium in vivo. *Proc. Natl. Acad. Sci. USA* **92**, 7809-7813.

GURBINDO, C, RUSSO, P, SABBAH, S, LOHOUES, MJ & SEIDMAN, E (1993) Interleukin-2 activity of colonic lamina propria mononuclear cells in a rat model of experimemtal colitis. *Gastroenterology* **104**, 964-972.

GUSTAFSON, C & TAGESSON, C (1990) Phospholipase C from Clostiridium perfingens stimulates phospholipase A2-mediated arachidonic acid release in cultured intestinal cell lines (INT 407). *Scand. J. Gastroenterol.* **25**, 363-371.

GUTIERREZ, HH, PITT, BR, SCHARZ, M, WATKINS, SC, LOWENSTEIN, C, CANIGGIA, I, CHUMLEY, P & FREEMAN, BA (1995) Pulmonary alveolar epithelial inducible NO synthase gene expression: regulation by inflammatory mediators. *Am. J. Phys.* **268**, L501-L508.

HADLEY, TJ, LU, ZH, WASNIOWSKA, K, MARTIN, AW, PEIRER, SC, HESSELGESSER, J & HOPUK, R (1994) Postcapillary venule endothelial cells in kidney express a multispecific chemokine receptor that is structurally and functionally identical to the erythroid isoform, which is the Duffy blood group antigen. *J. Clin. Invest.* **94**, 985-991.

HALSTENSEN, TS, MOLLNES, TE, FAUSA, O & BRANDTZAEG, P (1989) Deposits of terminal complement complex (TCC) in muscularis mucosae and submucosal vessels in ulcerative colitis and Crohn's disease of the colon. *Gut* **30**, 361-366.

HALSTENSEN, TS, MOLLNES, TE, GARVED, P, FAUSA, O & BRANDTZAEG, P (1990) Epithelial deposition of immunoglobulin G1 and activated complement (C3b and terminal complement complex) in ulcerative colitis. *Gastroenterology* **98**, 1264-1271.

HARRIES, AD, BAIRD, A & RHODES, J (1982) Non smoking: a feature of ulcerative colitis. *Br. Med. J.* **284**, 706

HARRIS, RR, CARTER, GW, BELL, RL, MOORE, JL & BROOKS, DW (1997) Clinical activity of leukotriene inhibitors. *Int. J. Immunopharmacol.* **17**, 147-156.

HARTLEY, MG, HUDSON, MJ, SWARBRICK, ET, GENT, AE, HELLIER, MD & GRACE, RH (1992) The rectal mucosa-associated microflora in patients with ulcerative colitis. *J. Med. Microbiol.* **36**, 96-103.

HATA, F, ISHII, T, KANADA, A, YAMANO, N, KATAOKA, T, TAKEUCHI, T & YAGASAKI, O (1990) Essential role of nitric oxide in descending inhibition in the rat proximal colon. *Biochem. Biophys. Res. Commun.* **172**, 1400-1406.

HAWKEY, CJ, DUBE, LM, ROUNTREE, LV, LINNEN, PJ & LANCASTER, JF (1997) A trial of zileuton versus mesalazine or placebo in the maintenance of remission of ulcerative colitis. *Gastroenterology* **112**, 718-724.

HEDGES, S, SVENSSON, M & SVANBORG, C (1992) Interleukin-6 response of epithelial cell lines to bacterial stimulation in vitro. *Infect. Immun.* **60**, 1295-1301.

HERBERT, JM, SAVI, P, LAPLACE, MC, LALE, A, DOL, F, DUMAS, A, LABIT, C & MINTY, A (1993) IL-4 and IL-13 exhibit comparable abilities to reduce pyrogen-induced expression of procoagulant activity in endothelial cells and monocytes. *FEBS Letts.* **328**, 268-270.

HIBBS, JB, TAINTOR, RR & VAVRIN, Z (1987a) Macrophage cytotoxicity: Role for L-arginine deiminase and amino nitrogen oxidation to nitrite. *Science* **235**, 473-476.

HIBBS, JB, VAVRIN, Z & TAINTOR, RR (1987b) L-Arginine is required for the expression of the activated macrophage effector machanism causing selective metabolic inhibition in target cells. *J. Immunol.* **138**, 550-565.

HOANG, P, CROTTY, B, DALTON, HR & JEWELL, DP (1992) Epithelial cells bearing class II molecules stimulate allogenic human colonic intraepithelial lymphocytes. *Gut* **33**, 1089-1093.

HOLLANDER, D (1992) The intestinal permeability barrier. A hypothesis as to its regulation and involvement in Crohn's disease. *Scand. J. Gastroenterol.* **27**, 721-726.

HOLLEN, CW, HENTHORN, J, KOJIOL, JA & BURNSTEIN, SA (1991) Elevated serum interleukin-6 levels in patients with reactive thrombocytosis. *Br. J. Haematol.* **79**, 286-290.

HOLMES, WE, LEE, J, KUANG, W-J, RICE, GC & WOOD, WI (1991) Structure and functional expression of a human interleukin-8 receptor. *Science* **253**, 1278-1280.

HOMMES, DW, MEENAN, J, DEHAAS, M, TENKATE, FJW, VONDEMBORNE, AEGK, TYTGAT, GNJ & VANDEVENTER, SJH (1996) Soluble Fc gamma receptor-III (CD 16) and eicosanoid concentrations in gut lavage fluid from patients with inflammatory bowel disease. Refluction of mucosal inflammation. *Gut* **38**, 564-567.

HOOGEWERF, AJ, BLACK, D, PROUDFOOT, AEI, WELLS, TNC & POWER, CA (1996) Molecular cloning of murine CC CKR-4 and high affinity binding of chemokines to murine and human CC CKR-4. *Biochem. Biophys. Res. Commun.* **218**, 337-343.

HORNQUIST, CE, LU, X, ROGERS-FANI, PM, RUDOLPH, U, SHAPPELL, S, BIRNBAUMER, L & HARRIMAN, GR (1997) Gai2-deficient mice with colitis exhibit a local increase in memory $CD4^+$ T cells and proinflammatory Th1-type cytokines. *J. Immunol.* **158**, 1068-1077.

HSU, D-H, DE WAAL MALEFYT, R, FIORENTINO, DF, DANG, M-N, VIEIRA, P, DE VRIES, J, SPITS, H, MOSMANN, TR & MOORE, KW (1990) Expression of interleukin-10 activity by Epstein-Barr virus proteinBCRF1. *Science* **250**, 830-832.

HUBER, AR, KUNKEL, SL, TODD, RF & WEISS, SJ (1991) Regulation of transendothelial neutrophil migration by endogenous interleukin-8. *Science* **254**, 99-102.

HULTEN, L, LINGHAGEN, J, LUNDGREN, O, FASTH, S & AHREN, C (1977) Regional blood flow in ulcerative colitis and Crohn's disease. *Gastroenterology* **72**, 388-396.

HUTCHESON, I, WHITTLE, BJR & BOUGHTON-SMITH, NK (1990) Role of nitric oxide in maintaining vascular integrity in endotoxin-induced acute intestinal damage in the rat. *Br. J. Pharmacol.* **101**, 815-820.

HYAMS, JS, FITZGERALD, JE, TREEM, WR, WYZGA, N & KREUTZER, DL (1993) Relationship of functional and antigenic interleukin 6 to disease activity in inflammatory bowel disease. *Gastroenterology* **104**, 1285-1292.

ISAACS, KL, SARTOR, RB & HASKILL, S (1992) Cytokine messenger RNA profiles in inflammatory bowel disease mucosa detected by polymerase chain reaction amplification. *Gastroenterology* **103**, 1587-1595.

IZZO, RS, WITKON, K, CHEN, AI, HADJIYANE, C, WEINSTEIN, MI & PELLECCHIA, C (1992) Interleukin-8 and neutrophil markers in colonic mucosa from patients with ulcerative colitis. *Am. J. Gastroenterol.* **87**, 1447-1452.

JANSSENS, SP, SHIMOUCH, A, QUERTERMOUS, T, BLOCH, DB & BLOCH, KD (1992) Cloning and expression of a cDNA encoding human endothelium-derived relaxing factor/nitric oxide synthase. *J. Biol. Chem.* **267**, 14519-14522.

JARNEROT, G (1996) Future aspects on inflammatory bowel disease. *Scand. J. Gastroenterol.* **31**, 87-90.

JIANG, Y, BELLER, DI, FRENDL, G & GRAVES, D (1992) Monocyte chemoattractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. *J. Immunol.* **148**, 2423-2428.

JORDAN, NJ, WATSON, ML & WESTWICK, J (1996) Differential effects of protein kinase C inhibitors on chemokine production in human synovial fibroblasts. *Br. J. Pharmacol.* **117**, 1245-1253.

JORENS, PG, VERMEIRE, PA & HERMAN, AG (1993) L-arginine dependent nitric oxide synthase. A new metabolic pathway in the lung and airways. *Eur. Respir. J.* **6**, 258-266.

JORENS, PG, MATTHYS, KE & BULT, H (1995) Modulation of nitric oxide synthase activity in macrophages. *Med. Inflammation* **4**, 75-89.

JUNG, HC, ECKMANN, L, YANG, SK, PANJA, A, FIERER, J, MORZYCKA-WROBLEWSKA, E & KAGNOFF, MF (1995) A distinct array of proinflammatory cytokines is expressed in human colon epithelial cells in response to bacterial invasion. *J. Clin. Invest.* **95**, 55-65.

JUNQUERO, DC, SCOTT-BURDEN, T, SCHINI, VB & VANHOUTTE, PM (1992) Inhibition of cytokine-induced nitric oxide production by transforming growth factor-b1 in human smooth muscle cells. *J. Physiol.* **454**, 451-465.

KAHN, R, LOHLER, J, RENNICK, D, RAJEWSKY, K & MALLER, W (1993) Interleukin-10-deficient mice develop chronic enterocolitis. *Cell* **75**, 263-274.

KAISERLIAN, D, RIGAL, D, ABELLO, J & REVILLARD, JP (1991) Expression, function and regulation of the intercellular adhesion molecule-1 (ICAM-1) on human intestinal epithelial cell lines. *Eur. J. Immunol.* **21**, 2415-2421.

KAM, L, PIZARRO, TT & COMINELLI, F (1995) Cytokines and chemokines in inflammatory bowel disease. *Curr. Opin. Gastroenterol.* **11**, 305-309.

KAMEYOSHI, Y, DÖRSCHNER, A, MALLET, AI, CHRISTOPHERS, E & SCHRÖDER, J-M (1992) Cytokine RANTES released by thrombin-stimulated platelets is a potent attractant for human eosinophils. *J. Exp. Med.* **176**, 587-592.

KANE, SP & VINCENTI, AC (1979) Mucosal enzymes in human inflammatory bowel disease with reference to neutrophil granulocytes as mediators of tissue injury. *Clin. Sci.* **57**, 295-303.

KARPUS, WJ, LUKACS, NW, KENNEDY, KJ, SMITH, WS, HURST, SD & BARRETT, TA (1997) Differential CC chemokine induced enhancement of T helper cell cytokine production. *J. Immunol.* **158**, 4129-4136.

KASAMA, T, STRIETER, RM, LUKACS, NW, BURDICK, MD & KUNKEL, SL (1994) Regulation of neutrophil-derived chemokine expression by IL-10. *J. Immunol.* **152**, 3559-3569.

KATSCHINSKI, B, FINGERLE, D, SCHERBAUM, B & GOEBELL, H (1993) Oral contraceptive use and cigarette smoking in Crohn's disease. *Dig. Dis. Sci.* **38**, 1596-1600.

KEEGAN, AD, JOHNSTON, JA, TORTOLANI, PJ, MCREYNOLDS, LJ, KINZER, C, O'SHEA, JJ & PAUL, WE (1995) Similarities and differences in signal transduction by interleukin 4 and interleukin 13: Analysis of Janus kinase activation. *Proc. Natl. Acad. Sci. USA* **92**, 7681-7685.

KELLY, CP, KEATES, S, SIEGENBERG, D, LINEVSKY, JK, POTHOULAKIS, C & BRADY, HR (1994) IL-8 secretion and neutrophil activation by HT-29 colonic epithelial cells. *Am. J. Physiol. Gastrointest. Liver Physiol.* **267**, G991-G997.

KELNER, GS, KENNEDY, J, BACON, KB, KLEYENSTEUBER, S, LARGAESPADA, DA, JENKINS, NA, COPELAND, NG, BAZAN, JF, MOORE, KW, SCHALL, TJ & ZLOTNIK, A (1994) Lymphotactin: A cytokine that represents a new class of chemokine. *Science* **266**, 1395-1399.

KENNEDY, J, KELNER, GS, KLEYENSTEUBER, S, SCHALL, TJ, WEISS, MC, YSSEL, H, SCHNEIDER, PV, COCKS, BJ, BACON, KB & ZLOTNIK, A (1995) Molecular cloning and functional characterization of human lymphotactin. *J. Immunol.* **155**, 203-209.

KERANEN, U, JARVINEN, H, KARKKAINEN, P, KIVILUOTO, T, KIVILAASKO, E & SOINILA, S (1996) Substance P-, an underlying factor for pouchitis. Prospective study of substance P-immunoreactive and vasoactive intestinal polypeptide immunoreactive innervation and mast cells. *Dig. Dis. Sci.* **41**, 1665-1671.

KIM, YI (1996) Can fish oil maintain Crohn's disease in remission. *Nutrit. Rev.* **54**, 248-252.

KIMURA, M, MASUDA, T, HIWATASHI, N, TOYOTA, T & NAGURA, H (1994) Changes in neuropeptide containing nerves in human colonic mucosa with inflammatory bowel disease. *Pathol. Int.* **44**, 624-634.

KING, T (1995) Meta-analysis of enteral nutrition as a primary treatment of active Crohn's disease; Comments. *Clin. Nutrit.* **14**, 388-389.

KITAGAWA, H, TAKEDA, F & KOHEI, H (1990) Effect of endothelium derived relaxing factor on the gastric lesion induced by HCl in rats. *J. Pharmacol. Exp. Ther.* **253**, 1133-1137.

KITAURA, M, NAKAJIAMA, T, IMAI, T, HARADA, S, COMBADIERE, C, TIFFANY, HL, MURPHY, PM & YOSHIE, O (1996) Molecular cloning of human eotoxin, an eosinophil-selective C-C chemokine, and identification of a specific eosiniphil eotoxin receptor. C-C chemokine receptor 3. *J. Biol. Chem.* **271**, 7725-7730.

KNOWLES, RG & MONCADA, S (1994) Nitric oxide synthases in mammals. *Biochem J.* **298**, 249-258.

KOBAYASHI, K, ATOH, M, KONOEDA, Y, YAGITA, A, INOKO, H & SEKIGUCHI, S (1990) HLA-DR, DQ and T cell antigen receptor constant beta genes in Japanese patients with ulcerative colitis. *Clin. Exp. Immunol.* **80**, 400-403.

KOCH, AE, POLVERINI, PJ, KUNKEL, SL, HARLOW, LA, DIPETRO, LA, ELNER, VM, ELLNER, SG & STRIETER, RM (1992) Interleukin 8 (IL-8) is a macrophage derived mediator of angiogenesis. *Science* **258**, 1798-1801.

KOIZUMI, M, KING, N, LOBB, R, BENJAMIN, C & PODOLSKY, DK (1992) Expression of vascular adhesion melecules in inflammatory bowel disease. *Gastroenterology* **103**, 840-847.

KOLB, JP, PAUL-EUGENE, N, DAMAIS, C, YAMAOKA, K, DRAPIER, JC & DUGAS, B (1994) Interleukin-4 stimulates cGMP production by IFN-gamma-activated human monocytes. *J. Biol. Chem.* **269**, 9811-9816.

KOLIOS, G, JORDAN, NJ, LEITHEAD, J, ROBERTSON, DAF & WESTWICK, J (1997a) Differential effect of IL-13 on C-X-C and C-C chemokine expression by colonic epithelial cells. *Gastroenterology* **112**, A1017

KOLIOS, G, ROONEY, N, MURPHY, CT, ROBERTSON, DAF & WESTWICK, J (1997b) Expression of inducible nitric oxide (iNOS) activity in human colon epithelial cells: Modulation by T-lymphocyte derived cytokines. *Gut* **in press**.

KOLIOS, G & NAKOS, A (1995) Cytokines in inflammatory bowel disease. *Hell. J. Gastroenterol.* **8**, 117-125.

KOUTROUBAKIS, I, MANOUSOS, ON, MEUWISSEN, SGM & PENA, AS (1996) Enviromental risk factors in inflammatory bowel disease. *Hepato-Gastroenterol.* **43**, 381-393.

KUBES, P & WALLACE, JL (1995) Nitric oxide as a mediator of gastrointestinal mucosal injury. *Med. Inflamm.* **4**, 397-405.

KUCHARZIK, T, LUGERING, N, WEIGELT, H, ADOLF, M, DOMSCHKE, W & STOLL, R (1996a) Immunoregulatory properties of IL-13 in patients with inflammatory bowel disease; comparison with IL-4 and IL-10. *Clin. Exp. Immunol.* **104**, 483-490.

KUCHARZIK, T, LUGERING, N, WEIGELT, H, ADOLF, M, DOMSCHKE, W & STOLL, R (1996b) Immunoregulatory properties of IL-13 in patients with inflammatory bowel disease. Comparison with IL-4 and IL-10. *Clin. Exp. Immunol.* **104**, 483-490.

KUCHARZIK, T, LUGERING, N, ADOLF, M, DOMSCHKE, W & STOLL, R (1997a) Synergistic effect of immunoregulatory cytokines on peripheral blood monocytes from patients with inflammatory bowel disease. *Dig. Dis. Sci.* **42**, 805-812.

KUCHARZIK, T, LUGERING, N, STOLL, R & DOMSCHKE, W (1997b) IL-4, IL-10, and IL-13 reveal strong capacity to downregulate MCP-1 and GM-CSF in activated intestinal epithelial cells. *Gut* **40**, F296

KUO, PC & SCHROEDER, RA (1995) The emerging multifaceted roles of nitric oxide. *Ann. Surg.* **221**, 220-235.

KVALE, D & BRANDTZAEG, P (1995) Constitutive and cytokine induced expression of HLA molecules, secretory component, and intercellular adhesion molecule-1 is modulated by butyrate in the colonic epithelial cell line HT-29. *Gut* **36**, 737-742.

KWON, NS, STUEHR, DJ & NATHAN, CF (1991) Inhibition of tumour cell ribonucleotide reductase by macrophage-derived nitric oxide. *J. Exp. Med.* **174**, 761-767.

LAEMMLI, UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685.

LAMAS, S, MARSDEN, PA, LI, GK, TEMPST, P & MICHEL, T (1992) Endothelial nitric oxide synthase: molecular cloning and characterization of a distinct, constitutive, enzyme isoform. *Proc. Natl. Acad. Sci. USA* **89**, 6348-6352.

LARSEN, CG, ANDERSON, AO, APPELLA, E, OPPENHEIM, JJ & MATSUSHIMA, K (1989) The neutrophil-activating protein (NAP-1) is also chemotactic for T lymphocytes. *Science* **243**, 1464-1466.

LASKIN, JD, HECK, DE & LASKIN, DL (1994) Multifunctional role of nitric oxide in inflammation. *TIEM* **5**, 377-382.

LAURITSEN, K, LAURSEN, LS & BUKHAVE, K (1989) Inflammatory intermediaries in inflammatory bowel disease. *Int. J. Colorect. Dis.* **4**, 75-90.

LEE, SC, DICKSON, DW, LIU, W & BROSNAN, CF (1993) Induction of nitric oxide synthase activity in human astrocytes by interleukin-1 beta and interferon-gamma. *J. Neuroimmunol.* **46**, 19-24.

LEONARD, EJ & YOSHIMURA, T (1990) Human monocyte chemoattractant protein-1 (MCP-1). *Immunol. Today* **11**, 97-101.

LESKO, SM, KAUFMAN, DW, ROSENBERG, L, HELMRICH, SP, MILLER, DR, STOLLEY, PD & SHAPIRO, S (1985) Evidence for an increased risk of Crohn's disease in oral contraceptive users. *Gastroenterology* **89**, 1046-1049.

LICHTMAN, SN & SARTOR, RB (1993) Examining the role of inflammatory cytokines in chronic inflammatory bowel disease. *J. Pediatr. Gastroenterol. Nutr.* **16**, 239-240.

LIEBERMAN, BY, FIOCCHI, C, YOUNGMAN, KR, SAPATNAKER, WK & PROFFIT, MR (1988) Interferon-gamma production by human intestinal lamina propria mononuclear cells. Decreased levels in inflammatory bowel disease. *Dig. Dis. Sci.* **47**, 1528-1536.

LIEW, FY (1995) Interactions between cytokines and nitric oxide. *Adv. Neuroimmun.* **5**, 201-209.

LIEW, FY, LI, Y, SEVERN, A, MILLOTT, S, SCHMIDT, J, SALTER, M & MONCADA, S (1991) A possible novel pathway of regulation by murine T-helper type-2 (Th2) cells of a Th1 cell activity via the modulation of the induction of nitric oxide synthase on macrophages. *Eur. J. Immunol.* **21**, 2489-2494.

LIHBRODY, L, POWELL, SR, COLLIER, KP, REDDY, GM, CERCHIA, R, KAHN, E, WEISSMAN, GS, KATZ, S, FLOYD, RA, MCKINLEY, MJ, FISHER, SE & MULLIN, GE (1996) Increased oxidative stress and decreased antioxidant defences in mucosa of inflammatory bowel disease. *Dig. Dis. Sci.* **41**, 2078-2086.

LINDBERG, E, JARNEROT, G & HUITFELDT, B (1992) Smoking in Crohn's disease; Effect on localization and clinical course. *Gut* **33**, 779-782.

LINDBERG, E, SODERHOLM, JD, OLAISON, G, TYSK, C & JARNEROT, G (1995) Intestinal permeability to polyethylene glycols in monozygotic twins with Crohn's disease. *Scand. J. Gastroenterol.* **30**, 780-783.

LINDEMAN, RJ, WEINSTEIN, L, LEVITAN, R & PATTERSON, JE (1967) Ulcerative colitis and intestinal salmonellosis. *Am. J. Med.* **254**, 855-861.

LINDLEY, IJD, WESTWICK, J & KUNKEL, SL (1993a) Nomenclature announcement - the chemokines. *Immunol. Today* **14**, 24

LINDLEY I.J.D., WESTWICK J. & KUNKEL S.L. (1993b) *The chemokines: Biology of the inflammmatory peptide supergene family (II) Advances in Experimental Medicine and Biology vol 351.* New York: Plenum. pp. 1-223.

LO, SK, DETMERS, PA, LEVIN, SM & WRIGHT, SD (1989) Transient adhesion of neutrophils to endothelium. *J. Exp. Med.* **169**, 1779-1793.

LOETSCHER, M, GERBER, B, LOETSCHER, P, JONES, SA, PIALI, L, CLARKLEWIS, I, BAGGIOLINI, M & MOSER, B (1996) Chemokine receptor specific for IP-10 and MIG; Structure, function, and expression in activated T-lymphocytes. *J. Exp. Med.* **184**, 963-969.

LOETSCHER, P, SEITZ, M, CLARK-LEWIS, I, BAGGIOLINI, M & MOSER, B (1994) Monocyte chemotactic proteins MCP-1, MCP-2, and MCP-3 are major attractants for human $CD4^+$ and $CD8^+$ T lymphocytes. *FASEB J.* **8**, 1055-1060.

LOWENSTEIN, CJ, GLATT, CS, BREDT, DS & SNYDER, SH (1992) Cloned and expressed macrophage nitric oxide synthase contrasts with the brain enzyme. *Proc. Natl. Acad. Sci. USA* **89**, 6711-6715.

LOWES, JR, RADWAN, P, PRIDDLE, JD & JEWELL, DP (1992) Characterisation and quantification of mucosal cytokine that induces epithelial histocompatibility locus antigen-DR expression in inflammatory bowel disease. *Gut* **33**, 315-319.

LU, L, MACKAY, C & WERSHIL, BK (1997) Pro-inflammatory cytokines induce the expression of eotoxin in Caco-2 intestinal epithelial cells. *Gastroenterology* **112**, A1029

LUNDBERG, JON, HELLSTROM, PM, LUNDBERG, JM & ALVING, K (1994a) Greatly increased luminal nitric oxide in ulcerative colitis. *Lancet* **344**, 1673-1674.

LUNDBERG, JON, WEITZBERG, E, LUNDBERG, JM & ALVING, K (1994b) Intragastric nitric oxide production in humans: measurmants in expelled air. *Gut* **35**, 1543-1546.

LUNDBERG, JON, WEITZBERG, E, NORDVALL, SL, KUYLENSTIERNA, R, LUNDBERG, JM & ALVING, K (1994c) Primarily nasal origin of exhaled nitric oxide and absence in Kartagener's syndrome. *Eur. Respir. J.* **7**, 1501-1504.

MACDONALD, TT & MURCH, SH (1994) Aetiology and pathogenesis of chronic inflammatory bowel disease. *Bailliere's Clinical Gastroenterology* **8**, 1-34.

MACK, DR, LAU, AS & SHERMAN, PM (1992) Systemic tumor necrosis factor-alpha production in experimental colitis. *Dig. Dis. Sci.* **37**, 1738-1745.

MACNAUGHTON, WK, CIRINO, G & WALLACE, JL (1989) Endothelium relaxing factor (nitric oxide) has protective actions in the stomach. *Life Sci.* **4589**, 1869-1876.

MACNAUGHTON, WK (1993) Nitric oxide-donating compounds stimulate electrolyte transport in the guinea pig intestine in vitro. *Life Sci.* **53**, 585-593.

MACPHERSON, A, KHOO, UY, FORGACS, I, PHILPOTTHOWARD, J & BJARNASON, I (1996) Mucosal antibodies in inflammatory bowel disease are directed against intestinal bacteria. *Gut* **38**, 365-375.

MADRETSMA, GS, DONZE, GJ, VANDIJK, APM, TAK, CJAM, WILSON, JHP & ZIJLSTRA, FJ (1996) Nicotine inhibits the in vitro production of interleukin-2 and TNF-a by human mononuclear cells. *Immunopharmacology* **35**, 47-51.

MADRETSMA, S, WOLTERS, LMM, VANDIJK, JPM, TAK, CJAM, FEYERABEND, C, WILSON, JHPW & ZIJLSTRA, FJ (1996) In vivo effect of nicotine on cytokine production by human nonadherent mononuclear cells. *Eur. J. Gastroenterol. Hepatol.* **8**, 1017-1020.

MAHIDA, YR, PATEL, S, GIONCHETTI, P, VAUX, D & JEWELL, DP (1989a) Macrophage subpopulations in lamina propria of normal and inflamed colon and terminal ileum. *Gut* **30**, 826-834.

MAHIDA, YR, WU, K & JEWELL, DP (1989b) Enhanced production of interleukin 1-b by mononuclear cells isolated from mucosa with active ulcerative colitis of Crohn's disease. *Gut* **30**, 835-838.

MAHIDA, YR, CESKA, M, EFFENBERGER, F, KURLAK, L, LINDLEY, I & HAWKEY, CJ (1992) Enhanced synthesis of neutrophil-activating peptide-1/interleukin-8 in active ulcerative colitis. *Clin. Sci.* **82**, 273-275.

MAIER, R, BILBE, G, REDISKE, J & LOTZ, M (1994) Inducible nitric oxide synthase from human articular chondrocytes: cDNA cloning and analysis of mRNA expression. *Biochim. Biophys. Acta* **1208**, 145-150.

MARFAING-KOKA, A, DEVERGNE, O, GORGONE, G, PORTIER, A, SCHALL, TJ, GALANAUD, P & EMILIE, D (1995) Regulation of the production of the RANTES chemokine by endothelial cells: Synergistic induction by IFN-gamma plus TNF-a and inhibition by IL-4 and IL-13. *J. Immunol.* **154**, 1870-1878.

MAYER, L, EISENHARDT, D, SALOMON, P, BAUER, W, PLOUS, R & PICCINI, L (1991) Expression of class II molecules on intestinal epithelial cells in humans. Differences between normal and inflammatory bowel disease. *Gastroenterology* **100**, 3-12.

MAZZUCCHELLI, L, HAUSER, C, ZGRAGGEN, K, WAGNER, H, HESS, M, LAISSUE, JA & MUELLER, C (1994) Expression of interleukin-8 gene in inflammatory bowel disease is related to the histological grade of active inflammation. *Am. J. Pathol.* **144**, 997-1007.

MAZZUCCHELLI, L, HAUSER, C, ZGRAGGEN, K, WAGNER, HE, HESS, MW, LAISSUE, JA & MUELLER, C (1996a) Differential *in situ* expression of the genes encoding the chemokines MCP-1 and RANTES in human inflammatory bowel disease. *J. Pathol.* **178**, 201-206.

MAZZUCCHELLI, L, LOETSCHER, P, KAPPELER, A & UGUCCIONI, M (1996b) Monocyte chemoattractant protein-1 gene expression in prostatic hyperplasia and prostate adenocarcinoma. *Am. J. Pathol.* **149**, 501-509.

MCCAIN, RW, DESSYPRIS, EN & CHRISTMAN, JW (1993) Granulocyte/macrophage colony-stimulating factor stimulates human polymorphonuclear leukocytes to produce interleukin-8 in vitro. *Am. J. Respir. Cell Mol. Biol.* **8**, 28-34.

MCCALL, TB, BOUGHTON-SMITH, NK, PALMER, RMJ, WHITTLE, BJR & MONCADA, S (1989) Synthesis of nitric oxide from L-arginine by neutrophils. Release and interaction with superoxide anion. *Biochem. J.* **261**, 293-296.

MCCALL, TB, PALMER, RMJ & MONCADA, S (1992) Interleukin-8 inhibits the induction of nitric oxide synthase in rat peritoneal neutrophils. *Biochem. Biophys. Res. Commun.* **186**, 680-685.

MCCARTHY, PL (1994) Down-regulation of cytokine action. *Baillieres Clin. Haematol.* **7**, 153-177.

MCKAY, DM & PERDUE, MH (1993a) Intestinal epithelial function: The case for immunophysiological regulation. Cells and mediators (First of two parts). *Dig. Dis. Sci.* **38**, 1377-1387.

MCKAY, DM & PERDUE, MH (1993b) Intestinal epithelial function: The case for immunophysiological regulation: Implications for disease (second of two parts). *Dig. Dis. Sci.* **38**, 1735-1745.

MCKENZIE, ANJ, CULPEPPER, JA, DE WAAL MALEFYT, R, BRIERE, F, PUNNONEN, J, AVERSA, G, SATO, A, DANG, W, COCKS, BG, MENON, S, DE VRIES, JE, BANCHEREAU, J & ZURAWSKI, G (1993) Interleukin-13, a T-cell-derived cytokine that regulates human monocyte and B-cell function. *Proc. Natl. Acad. Sci. USA* **90**, 3735-3739.

MCKENZIE, SJ, BAKER, MS, BUFFINTON, GD & DOE, WF (1996) Evidence of oxidant-induced injury to epithelial cells during inflammatory bowel disease. *J. Clin. Invest.* **98**, 136-141.

MEENAN, J, GROOL, TA, HOMMES, DW, DIJKHUIZEN, S, TENKATE, FJ, WOOD, M, WHITTAKER, M, TYTGAT, GN & VANDEVENDER, SJ (1996) Lexipafant (BB-

882), a platelet-activating-factor receptor antagonist, ameliorates mucosal inflammation in an animal model of colitis. *Eur. J. Gastroenterol. Hepatol.* **8**, 569-573.

MIDDLETON, SJ, SHORTHOUSE, M & HUNTER, JO (1993a) Increased nitric oxide synthesis in ulcerative colitis. *Lancet* **341**, 465-466.

MIDDLETON, SJ, SHORTHOUSE, M & HUNTER, JO (1993b) Relaxation of distal colonic circular smooth muscle by nitric oxide derived from human leukocytes. *Gut* **34**, 814-817.

MILLER, MD & KRANGEL, MS (1992) Biology and biochemistry of the chemokines: a family of chemotactic and inflammatory cytokines. *Crit. Rev. Immunol.* **12**, 17-46.

MINTY, A, CHALON, P, DEROCQ, J-M, DUMONT, X, GUILLEMOT, J-C, KAGHAD, M, LABIT, C, LEPLATOIS, P, LIAUZAN, P, MILOUX, B, MINTY, C, CASSE;;AS, P, LOISON, G, LUPKER, J, SHIRE, D, FERRARA, P & CAPUT, D (1993) Interleukin-13 is a new human lymphokine regulating inflammatory and immune responses. *Nature* **362**, 248-250.

MISHINA, D, KATSEL, P, BROWN, ST, GILBERTS, ECAM & GREENSTEIN, RJ (1996) On the etiology of Crohn's disease. *Proc. Natl. Acad. Sci. USA* **93**, 9816-9820.

MISHKIN, S (1997) Dairy sensitivity, lactose malabsorption, and elimination diets in inflammatory bowel disease. *Am. J. Clin. Nutr.* **65**, 564-567.

MISKO, TP, SCHILLING, RJ, SALVEMINI, D, MOORE, WM & CURRIE, MG (1993) A flourometric assay for the measurement of nitrite in biological samples. *Anal. Biochem.* **214**, 11-16.

MITSUYAMA, K, TOYONAGA, A, SASAKI, E, WATANABE, K, TATEISHI, H, NISHIYAMA, T, SAIKI, T, IKEDA, H, TSURUTA, O & TANIKAWA, K (1994) IL-8 as an important chemoattractant for neutrophils in ulcerative colitis and Crohn's disease. *Clin. Exp. Immunol.* **96**, 432-436.

MIURA, M & HIWATASHI, N (1985) Cytokine production in inflammatory bowel disease. *J. Clin. Lab. immunol.* **18**, 81-86.

MOILANEN, E & VAPAATALO, H (1995) Nitric oxide in inflammation and immune response. *Ann. Med.* **27**, 359-367.

MOLINA Y VEDIA, L, MCDONALD, B, REEP, B, BRUNE, B, DI SILVIO, M, BILLIAR, TR & LAPETINA, EG (1992) Nitric oxide-induced S-nitrosylation of glyceraldehyde-3-phosphate dehydrogenase inhibits enzymatic activity and increases endogenous ADP-ribosylation. *J. Biol. Chem.* **267**, 24929-24932.

MONCADA, S, PALMER, RMJ & HIGGS, EA (1991) Nitric oxide: physiology, pathophysiology and pharmacology. *Pharmacol. Rev.* **43**, 109-142.

MONCADA, S & HIGGS, A (1993) The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**, 2002-2012.

MOORE, KW, O'GARRA, A, DE WAAL MALEFYT, R, VIEIRA, P & MOSMANN, TR (1993) Interleukin-10. *Annu. Rev. Immunol.* **11**, 165-190.

MORRIS, SM & BILLIAR, TR (1994) New insights into the regulation of inducible nitric oxide synthase. *Am. J. Physiol.* **266**, E829-E839.

MORTENSEN, PB, HOVE, H, CLAUSEN, MR & HOLTUG, K (1991) Fermentation to short-chain fatty acids and lactate in human feacal batch cultures. Intra- and inter-individual variations versus variations caused by changes in fermented saccharides. *Scand. J. Gastroenterol.* **26**, 1285-1294.

MOSMANN, TR & COFFMAN, RL (1989) Heterogeneity of cytokine secreting patterns and functions of helper T cells. *Adv. Immunol.* **46**, 111-117.

MOSTOV, KE, FRIEDLANDER, M & BLOBEL, G (1984) The receptor for trans-epithelial transport of IgA and IgM contains multiple immunoglobulin-like domains. *Nature* **308**, 37-43.

MUELLER, C, KNOFLACH, P & ZIELINSKI, CC (1990) T-cell activation in Crohn's disease. Increased levels of soluble interleukin 2 receptor antagonist in serum and in supernatants of stimulated peripheral blood mononuclear cells. *Gastroenterology* **98**, 693-646.

MULLIN, GE, LAZENBY, AJ, HARRIS, ML, BAYLESS, TM & JAMES, SP (1992) Increased interleukin-2 messenger RNA in the intestinal mucosal lesions of Crohn's disease and ulcerative colitis. *Gastroenterology* **102**, 1620-1627.

MURATA, Y, ISHIGURO, Y, ITOH, J, MUNAKATA, A & YOSHIDA, Y (1995) The role of pro-inflammatory and immunoregulatory cytokines in the pathogenesis of ulcerative colitis. *J. Gastroenterol.* **30**, 56-60.

MURPHY, PM & TIFFANY, HL (1991) Cloning of complementary DNA encoding a functional human interleukin-8 receptor. *Science* **253**, 1280-1283.

MUTCHNICK, MG, LEE, HH, HOLANDER, DL, HAYNES, GD & CHUA, DC (1988) Defective in vitro gamma interferon production and elevated serum immunoreactive thymocin b4 levels in patients with inflammatory bowel disease. *Clin. Immunol. Immunopathol.* **47**, 8492-8497.

MUZIO, M, RE, F, SIRONI, M, POLENTARUTTI, N, MINTY, A, CAPUT, D, FERRARA, P, MANTOVANI, A & COLOTTA, F (1994) Interleukin-13 induces the production of interleukin-1 receptor antagonist (IL-1ra) and the expression of the mRNA for the intracellular (keratinocyte) form of IL-1ra in human myelomonocytic cells. *Blood* **83**, 1738-1743.

NAKAMURA, M, SAITO, H, KASANUKI, J, TAMURA, Y & YOSHIDA, S (1992) Cytokine production in patients with inflammatory bowel disease. *Gut* **33**, 933-937.

NAKAYAMA, I, KAWAHARA, Y, TSUDA, T, OKUDA, M & YOKOYAMA, M (1994) Angiotensin II inhibits cytokine-stimulated inducible nitric oxide synthase expression in vascular smooth muscle cells. *J. Biol. Chem.* **269**, 11628-11633.

NEOTE, K, DIGREGORIO, D, MAK, JY, HORUK, R & SCHALL, TJ (1993) Molecular-cloning, functional expression, and signaling characteristics of a c-c chemokine receptor. *Cell* **72**, 415-425.

NEOTE, K, MAK, JY, KOLAKOWSKI, LF & SCHALL, TJ (1994) Functional and biochemical analysis of the cloned Duffy antigen identity with the red blood cell chemokine receptor. *Blood* **84**, 44-52.

NEWMAN, A & LAMBERT, JR (1990) Cambylobacter causing flareup in inflammatory bowel disease. *Lancet* **2**, 919

NICOLSON, AG, HAITES, NE, MCKAY, NG, WILSON, HM, MACLEOD, AM & BENJAMIN, N (1993) Induction of nitric oxide synthase in human mesangial cells. *Biochem. Biophys. Res. Commun.* **193**, 1269-1274.

NIELSEN, OH, KOPPEN, T, RUDIGER, N, HORN, T, ERIKSEN, J & KIRMAN, I (1996) Involvement of interleukin-4 and interleukin-10 in inflammatory bowel disease. *Dig. Dis. Sci.* **41**, 1786-1793.

NIELSEN, OH & RASK-MADSEN, J (1996) Mediators of inflammation in chronic inflammatory bowel disease. *Scand. J. Gastroenterol.* **31: Suppl 216**, 149-159.

NORTH, CS, ALPERS, DH, HELZER, JE, SPITZNAGEL, EL & CLOUSE, RE (1991) Do life stress events or deptession exacerbate inflammatory bowel disease? A prospective study. *Ann. Internal Med.* **114**, 381-386.

NOSTRANT, TT, KUMAR, NB & APPELMAN, HD (1987) Histopathology differentiates acute self-limited colitis from ulcerative colitis. *Gastroenterology* **92**, 318-328.

NUSSLER, AK, DI SILVIO, M, BILLIAR, TR, HOFFMAN, RA, GELLER, DA, SELBY, R, MADARIAGA, J & SIMMONS, RL (1992) Stimulation of the nitric oxide pathway in human hepatocytes by cytokines and endotoxin. *J. Exp. Med.* **176**, 261-264.

O'MORAIN, C, SMETHURST, P, LEVI, AJ & PETERS, TJ (1983) Biochemical analysis of enzyme markers of inflammation in rectal biopsies from patients with ulcerative colitis and Crohn's disease. *J. Clin. Pathol.* **36**, 1312-1316.

O'MORAIN, C, SEGAL, AW & LEVI, AJ (1984) Elemental diet as primary treatment of acute Crohn's disease: a controlled trial. *Br. Med. J.* **288**, 1859-1860.

OHSHIMA, H & BARTSCH, H (1994) Chronic infections and inflammatory processes as cancer risk factors. Possible role of nitric oxide in carcinogenesis. *Mutat. Res.* **305**, 253-264.

OKEEFE, SJD (1996) Nutrition and gastrointestinal disease. *Scand. J. Gastroenterol.* **31**, 52-59.

OPPENHEIM, JJ, ZACHARIAE, COC, MUIKADA, N & MATSUSHIMA, K (1991) Properties of the novel proinflammatory supergene "intercrine" cytokine family. *Annu. Rev. Immunol.* **9**, 617-648.

ORIISHI, T, SATA, M, TOYONAGA, A, SASAKI, E & TANIKAWA, K (1995) Evaluation of intestinal permeability in patients with inflammatory bowel disease using lactulose nad measuring antibodies to lipid-A. *Gut* **36**, 891-896.

OSWALD, IP, GAZZINELLI, RT, SHER, A & JAMES, SL (1992) IL-10 synergizes with IL-4 and transforming growth factor beta to inhibit macrophage cytotoxic activity. *J. Immunol.* **148(11)**, 3578-3582.

PALLONE, F & MONTELLEONE, G (1996) Regulatory cytokines in inflammatory bowel disease. *Aliment. Pharmacol. Therap.* **10**, 75-79.

PALMER, RMJ, HICKERY, MS, CHARLES, IG, MONCADA, S & BAYLISS, MT (1993) Induction of nitric oxide synthase in human chondrocytes. *Biochem. Biophys. Res. Commun.* **193**, 398-405.

PENA, AS, CRUSIUS, JBA, OUDKERK POOL, M, GRACIA CASANOVA, M, PALS, G, MEUWISSEN, SGM & GIPHART, MJ (1993) Genetics and epidemiology may contribute to understanding the pathogenesis of IBD; A new approach is now indicated. *Can. J. Gastroenterol.* **7**, 71-75.

PERSSON, PG, AHLBOM, A & HELLERS, G (1992) Diet and inflammatory bowel disease; A case control study. *Epidemiology* **3**, 47-52.

PFEILSCHIFTER, J (1991) Anti-inflammatory steroids inhibit cytokine induction of nitric oxide synthase in rat renal mesangial cells. *Eur. J. Pharmacol.* **195**, 179-180.

PFEILSCHIFTER, J, EBERHARDT, W, HUMMEL, R, KUNZ, D, MUHL, H, NITSCH, D, PLUSS, C & WALKER, G (1996) Therapeutic strategies for the inhibition of inducible nitric oxide synthase potential for a novel class of anti-inflammatory agents. *Cell Biol. Int.* **20**, 51-58.

PLAYFORD, RJ, HANBY, AM, PATEL, K & CALAM, J (1995) Influence of inflammatory bowel disease on the distrbution and concentration of pancreatic secretory trypsin inhibitor within the colon. *Am. J. Pathol.* **146**, 310-316.

PLEVY, SE, TARGAN, SR, YANG, HY, FERNANDEZ, D, ROTTER, JI & TOYODA, H (1996) Tumor necrosis factor microsatellites define a Crohn's disease associated haplotype on chromosome 6. *Gastroenterology* **110**, 1053-1060.

PODOLSKY, DK (1991) Inflammatory bowel disease. *N. Engl. J. Med.* **325**, 928-937.

PODOLSKY, DK & FOURNIER, DA (1988) Alterations in mucosal content of colonic glycoconjugates in inflammatory bowel disease defined by monoclonal antibodies. *Gastroenterology* **95**, 379-387.

POOL, MO, BOUMA, G, VISSER, JJ, KOLKMAN, JJ, TRAN, DD, MEUWISSEN, SGM & PEÑA, AS (1995) Serum nitrate levels in ulcerative colitis and Crohn's disease. *Scand. J. Gastroenterol.* **30**, 784-788.

POOLE, S, CUNHA, FQ, SELKIRK, S, LORENZETTI, BB & FERREIRA, SH (1995) Cytokine-mediated inflammatory hyperalgesia limited by interleukin-10. *Br. J. Pharmacol.* **115**, 684-688.

POWER, CA, MEYER, A, NEMETH, K, BACON, KB, HOOGEWERF, AJ, PROUDFOOT, AEI & WELLS, TNC (1995) Molecular cloning and functional expression of a novel CC chemokine receptor cDNA from a human basophilic cell line. *J. Biol. Chem.* **270**, 19495-19500.

POWER, CA & WELLS, TNC (1996) Cloning and characterization of human chemokine receptors. *TIPS* **17**, 209-213.

PROOST, P, WUYTS, A & VANDAMME, J (1996) The role of chemokines in inflammation. *Int. J. Clin. Lab. Res.* **26**, 211-223.

PRZEMIOSLO, RT & CICLITIRA, PJ (1996) Cytokines and gastrointestinal disease mechanisms. *Bailliere's Clinical Gastroenterology* **10**, 17-32.

PULLAN, RD, RHODES, J, GANESH, S, MANI, V, MORRIS, JS, WILLIAMS, GT, NEWCOMBE, RG, RUSSELL, MAH, FEYERABEND, C, THOMAS, GAO & SAWE, U (1994) Transdermal nicotine for active ulcerative colitis. *N. Engl. J. Med.* **330**, 811-815.

RAAB, Y, GERDIN, B, AHLSTEDT, S & HÄLLGREN, R (1993) Neutrophil mucosal involvement is accompanied by enhanced local production of interleukin-8 in ulcerative colitis. *Gut* **34**, 1203-1206.

RAAB, Y, SUNDBERG, C, HALLGREN, R, KNUTSON, L & GERDIN, B (1995) Mucosal synthesis and release of prostaglandin E(2) from activated eosinophils and macrophages in ulcerative colitis. *Am. J. Gastroenterol.* **90**, 614-620.

RACHMILEWITZ, D, KARMELI, F & OKON, E (1995a) Sulfhydryl blocker-induced rat colonic inflammation is ameliorated by inhibition of nitric oxide synthase. *Gastroenterology* **109**, 98-106.

RACHMILEWITZ, D, KARMELI, F, OKON, E & BURSZTYN, M (1995b) Experimental colitis is ameliorated by inhibition of nitric oxide synthase activity. *Gut* **37**, 247-255.

RACHMILEWITZ, D, STAMLER, JS, BACHWICH, D, KARMELI, F, ACKERMAN, Z & PODOLSKY, DK (1995c) Enhanced colonic nitric oxide generation and nitric oxide synthase activity in ulcerative colitis and Crohn's disease. *Gut* **36**, 718-723.

RADFORD-SMITH, D & JEWELL, DP (1996) Cytokines and inflammatory bowel disease. *Bailliere's Clinical Gastroenterology* **10**, 151-164.

RADOMSKI, MW, PALMER, RMJ & MONCADA, S (1990) Glucocorticoids inhibit the expression of an inducible, but not the constitutive, nitric oxide synthase in vascular endothelial cells. *Proc. Natl. Acad. Sci. USA* **87**, 10043-10047.

RAITHEL, M, MATEK, M, BAENKLER, HW, JORDE, W & HAHN, EG (1995) Mucosal histamine content and histamine secretion in Crohn's disease, ulcerative colitis and allergic enteropathy. *Int. Arch. Allergy Immunol.* **108**, 127-133.

RAMPTON, DS, MURDOCH, RD & SLADEN, GE (1980) Rectal mucosal histamine release in ulcerative colitis. *Clin. Sci.* **59**, 389-391.

RAMPTON, DS, SALMON, PR & CLARK, CG (1983) Nonspecific ulcerative colitis as a sequel to amoebic dysentery. *J. Clin. Gastroenterol.* **5**, 217-219.

RAMPTON, DS (1987) Nonsteroidal antiinflammatory drugs and the lower gastrointestinal tract. *Scand. J. Gastroenterol.* **22**, 1-4.

RAMPTON, DS & COLLINS, CE (1993) Thromboxanes in inflammatory bowel disease-pathogenic and therapeutic implications. *Aliment. Pharmacol. Therap.* **7**, 357-367.

RAMPTON, DS & HAWKEY, CJ (1984) Prostagladins and ulcerative colitis. *Gut* **25**, 1399-1413.

REILING, N, ULMER, AJ, ERNST, M, FLAD, HD & HAUSCHILDT, S (1994) Nitric oxide synthase: mRNA expression of different isoforms in human monocytes/macrophages. *Eur. J. Immunol.* **24**, 1941-1944.

REIMUND, JM, WITTERSHEIM, C, DUMONT, S, MULLER, CD, BAUMANN, R, POINDRON, P & DUCLOS, B (1996) Mucosal inflammatory cytokine production by intestinal biopsies in patients with ulcerative colitis and Crohn's disease. *J. Clin. Immunol.* **16**, 144-150.

REINECKER, HC, LOH, EY, RINGLER, DJ, MEHTA, A, ROMBEAU, JL & MACDERMOTT, RP (1995) Monocyte-chemoattractant protein 1 gene expression in intestinal epithelial cells and inflammatory bowel disease mucosa. *Gastroenterology* **108**, 40-50.

RENNICK, DM, FORT, MM & DAVIDSON, NJ (1997) Studies with IL-10 deficient mice: an overview. *J. Leukoc. Biol.* **61**, 389-396.

REUBI, JC, MAZZUCCHELLI, L & LAISSUE, JA (1994) Intestinal vessels express a high density of somatostatin receptors in human inflammatory bowel disease. *Gastroenterology* **106**, 951-959.

REYNOLDS, PD, MIDDLETON, SJ, HANSFORD, GM & HUNTER, JO (1995a) Nitric oxide in ulcerative colitis. *Lancet* **345**, 448-449.

REYNOLDS, PD, MIDDLETON, SJ, HUNTER, JO, FACER, P, BISHOP, A, EVANS, T & POLAK, JM (1995b) High expression of iNOS in colonic mucosa in ulcerative colitis. *Gastroenterology* **108(4)**, A903

RHODES, JM, BLACK, RR & SAVAGE, A (1988) Altered lectin binding by colonic epithelial glycoconjugates in ulcerative colitis and Crohn's disease. *Dig. Dis. Sci.* **33**, 1359-1363.

RIBBONS, KA, ZHANG, X-J, THOMPSON, JH, GREENBERG, SS, MOORE, WM, KORNMEIER, CM, CURRIE, MG, LERCHE, N, BLANCHARD, J, CLARK, DA & MILLER, MJS (1995) Potential role of nitric oxide in a model of chronic colitis in rhesus macaques. *Gastroenterology* **108**, 705-711.

RIORDAN, AM, HUNTER, JO & COWAN, RE (1993) Treatment of Crohn's disease by exclusion diet: East Anglian Multicentre Controlled Trial. *Lancet* **342**, 1131-1134.

ROBBINS, RA, BARNES, PJ, SPRINGALL, DR, WARREN, JB, KWON, OJ, BUTTERY, LDK, WILSON, AJ, GELLER, DA & POLAK, JM (1994a) Expression of inducible nitric oxide in human lung epithelial cells. *Biochem. Biophys. Res. Commun.* **203**, 209-218.

ROBBINS, RA, SPRINGALL, DR, WARREN, JB, KWON, OJ, BUTTERY, LDK, WILSON, AJ, ADCOCK, IM, RIVEROS-MORENO, V, MONCADA, S, POLAK, J & BARNES, PJ (1994b) Inducible nitric oxide synthaseis increased in murine lung epithelial cells by cytokine stimulation. *Biochem. Biophys. Res. Commun.* **198**, 835-843.

ROBERTS, WG, SIMON, TJ, BERLIN, RG, HAGGITT, RC, SNYDER, ES, STENSON, WF, HANAUER, SB, REAGAN, JE, CAGLIOLA, A, TANAKA, WK, SIMON, S & BERGER, ML (1997) Leukotrienes in ulcerative colitis: Results of a multicenter trial of a leukotriene biosyntheses inhibitor, MK-591. *Gastroenterology* **112**, 725-732.

ROEDIGER, WEW (1980a) The colonic epithelium in ulcerative colitis: an energy deficiency disease? *Lancet* **2**, 712-715.

ROEDIGER, WEW (1980b) Role of anaerobic bacteria in the metabolic welfare of the colonic mucosa in man. *Gut* **21**, 793-798.

ROGLER, G, DAIG, R, VOGL, D, SCHOLMERICH, J, GROSS, V & ANDUS, T (1997) Induction of the secretion of MCP-1 and MIP-1a in primary cultures of human intestinal epithelial cells by foetal calf serum. *Gastroenterology* **112**, A1076

ROLLINS, BJ, WALZ, A & BAGGIOLINI, M (1991) Recombinant human MCP-1/JE induces chemotaxis, calcium flux, and the respiratory burst in human monocytes. *Blood* **78**, 1112-1119.

ROLLINS, BJ (1997) Chemokines. *Blood* **90**, 909-928.

ROT, A (1992) Endothelial cell binding of NAP-1/IL-8: Role in neutrophil emigration. *Immunol. Today* **13**, 291-294.

ROWBOTHAM, DS, HOWDLE, PD & TREJDOSIEWICZ, LK (1995) Peripheral cell mediated immune response to mycobacterial antigens in inflammatory bowel disease. *Clin. Exp. Immunol.* **102**, 456-461.

RUPPIN, H, BAR-MEIR, S, SOERGEL, KH, WOOD, CM & SCHMITT, MG (1980) Absorption of short-chain fatty acids by the colon. *Gastroenterology* **78**, 1500-1507.

SAITOH, H, TAKAGAKI, K, NAKAMURA, T, MUNAKATA, A, YOSHIDA, Y & ENDO, M (1996) Characterization of mucin in whole gut lavage fluid obtained from patients with inflammatory bowel disease. *Dig. Dis. Sci.* **41**, 1768-1774.

SALIM, AS (1992) Role of oxygen-derived free radical scavengers in the management of reccurent attacks of ulcerative colitis: a new approach. *J. Lab. Clin. Med.* **119**, 710-719.

SAMSON, M, LABBE, O, MOLLEREAU, C, VASSART, G & PARMENTIER, M (1996) Molecular cloning and functional expression of a new human CC chemokine receptor gene. *Biochemistry* **35**, 3362-3367.

SANAHAN, F & ANTON, P (1988) Neuroendocrine modulation of the immune system. Possible implications for inflammatory bowel disease. *Dig. Dis. Sci.* **33**, 41s-49s.

SANDS, BE & PODOLSKY, DK (1996) Toward new questions in inflammatory bowel disease in 1995. *Curr. Opin. Gastroenterol.* **12**, 319-321.

SARTOR, RB (1994) Cytokines in intestinal inflammation: Pathophysiological and clinical considerations. *Gastroenterology* **106**, 533-539.

SARTOR, RB, DELACADENA, RA, GREEN, KD, STADNICKI, A, DAVIS, SW, SCHWAB, JH, ADAM, AA, RAYMOND, P & COLMAN, RW (1996a) Selective kallikrein-kinin system activation in inbred rats differentially susceptible to granulomatus enterocolitis. *Gastroenterology* **110**, 1467-1481.

SARTOR, RB, RATH, HC & SELLON, RK (1996b) Microbial factors in chronic intestinal inflammation. *Curr. Opin. Gastroenterol.* **12**, 327-333.

SATSANGI, J, WELSH, KI, BUNCE, M, JULIER, C, FARRANT, JM, BELL, JI & JEWELL, DP (1996) Contribution of genes of the major histocompatibility complex to susceptibility and disease phenotype in inflammatory boewl disease. *Lancet* **347**, 1212-1217.

SAURA, M, MARTINEZ-DALMAU, R, MINTY, A, PEREZ-SALA, D & LAMAS, S (1996) Interleukin-13 inhibits inducible nitric oxide expression in human mesangial cells. *Biochem J.* **313**, 641-646.

SAVERYMUTTU, SH, CAMILLERI, M, REES, H, LAVENDER, JP, HODGSON, HJ & CHADWICK, VS (1986) Indium^{111}-granulocyte scanning in the assessment of disease extent and disease activity in inflammatory bowel disease. *Gastroenterology* **90**, 1121-1128.

SCHALL, TJ, BACON, K, TOY, KJ & GOEDDEL, DV (1990) Selective attraction of monocytes and T lymphocytes of the memory phenotype by cytokine RANTES. *Nature* **347**, 669-671.

SCHALL, TJ (1994) The chemokines. In *The cytokine handbook.* 2nd ed. Ed. AnonymousAcademic Press Limited. pp. 419-457.

SCHMIDT, C, KOSCHE, E, BAUMEISTER, B & VETTER, H (1995) Arachidonic acid metabolism and intracellular calcium concentration in inflammatory bowel disease. *Eur. J. Gastroenterol. Hepatol.* **7**, 865-869.

SCHMIDT, C, FELS, T, BAUMEISTER, B & VETTER, H (1996) The effect of 5-aminosalicylate and para-aminosalicylate on the synthesis of prostaglandin E(2) and leukotriene B (4) in isolated colonic mucosal cells. *Curr. Med. Res. Opin.* **13**, 417-425.

SCHMITZ, H, FROMM, M, BODE, H, SCHOLZ, P, RIECKEN, EO & SCHULZKE, JD (1996) Tumor necrosis factor-a induces Cl^- and K^+ secretion in human distal colon driven by prostaglandin E(2). *Am. J. Physiol.* **34**, G669-G674.

SCHMOUDER, RL, STRIETER, RM, WIGGINS, RC, CHENSUE, SW & KUNKEL, SL (1992) In vitro and in vivo interleukin-8 production in human renal cortical epithelia. *Kidney Int.* **41**, 191-198.

SCHMOUDER, RL, STRIETER, RM & KUNKEL, SL (1993) Interferon-gamma regulation of human renal cortical epithelial cell-derived monocyte chemotactic peptide-1. *Kidney Int.* **44**, 43-49.

SCHMUCKER, DL, HEYWORTH, MF, OWEN, RL & DANIELS, CK (1996) Impact of aging on gastrointestinal mucosal immunity. *Dig. Dis. Sci.* **41**, 1183-1193.

SCHREIBER, S, HEINIG, T, PANZER, U, REINKING, R, BOUCHARD, A, STAHL, PD & RAEDLER, A (1995a) Impaired response of activated mononuclear phagocytes to interleukin 4 in inflammatory bowel disease. *Gastroenterology* **108**, 21-33.

SCHREIBER, S, HEINIG, T, THIELE, H-G & RAEDLER, A (1995b) Immunoregulatory role of interleukin 10 in patients with inflammatory bowel disease. *Gastroenterology* **108**, 1434-1444.

SCHUERER-MALY, C-C, ECKMANN, L, KAGNOFF, MF, FALCO, MT & MALY, FE (1994) Colonic epithelial cell lines as a source of interleukin-8: Stimulation by inflammatory cytokines and bacterial lipopolysaccharide. *Immunology* **81**, 85-91.

SCHUERMANN, GM, ABER-BISHOP, AE, FACER, P, LEE, JC, RAMPTON, DS, DORE, CJ & POLAK, JM (1993) Altered expression of cell adhesion molecules in uninvolved gut in inflammatory bowel disease. *Clin. Exp. Immunol.* **94**, 341-347.

SCHULTEBOCKHOLT, A, FINK, JG, MEIER, DA, OTTERSON, MF, TELFORD, GL, HOPP, K & KOCH, TR (1995) Expression of messenger RNA for vasoactive intestinal peptide in normal human colon and during inflammation. *Mol. Cell. Biochem.* **142**, 1-7.

SEITZ, R, LERCH, L, IMMEL, A & EGBRING, R (1995) D-dimer tests detect both plasmin and neutrophil elastase derived split products. *Ann. Clin. Biochem.* **32**, 193-195.

SELBY, WS, JANOSSY, G, MASON, DY & JEWELL, DP (1983) Expression HLA-DR antigens by colonic epithelium in inflammatory bowel disease. *Clin. Exp. Immunol.* **53**, 614-618.

SHANAHAN, F (1993) Pathogenesis of ulcerative colitis. *Lancet* **342**, 407-411.

SHERMAN, PA, LAUBACH, VE, REEP, BR & WOOD, ER (1993) Purification and cDNA sequence of an inducible nitric oxide synthase from a human tumour cell line. *Biochemistry* **32**, 11600-11605.

SIMMONDS, NJ & RAMPTON, DS (1993) Inflammatory bowel disease - a radical view. *Gut* **34**, 865-868.

SIMON, MR, GATMAITAN, BG, WEINSTOCK, JV, SALKELD, RM & WILLIAMS, BB (1983) Antiviral activity in sera of patients with Crohn's disease. *Am. J. Med. Sci.* **286**, 21-25.

SINCLAIR, R, ERIKSSON, AS, GRETZER, C, CASSUTO, J & THOMSEN, P (1993) Inhibitory effects of amide local anaesthetics on stimulus-induced human leucocyte metabolic activation, LTB4 release and IL-1 secretion in vitro. *Acta Anaesth. Scand.* **37**, 159-165.

SINGER, II, KAWKA, DW, SCOTT, S, WEIDNER, JR, MUMFORD, RA, RIEHL, TE & STENSON, WF (1996) Expression of inducible nitric oxide synthase and nitrotyrosine in colonic epithelium in inflammatory bowel disease. *Gastroenterology* **111**, 871-885.

SIRONI, M, SCIACCA, FL, MATTEUCCI, C, CONNI, M, VECCHI, A, BERNASCONI, S, MINTY, A, CAPUT, D, FERRARA, P, COLOTTA, F & MANTOVANI, A (1994) Regulation of endothelial and mesothelial cell function by interleukin-13: Selective induction of vascular cell adhesion molecule-1 and amplification of interleukin-6 production. *Blood* **84**, 1913-1921.

SMITH, KA (1988) Interleukin-2: Inception, impact, and implications. *Science* **240**, 1169-1176.

SOZZANI, S, LOCATI, M, ZHOU, D, RIEPPI, M, LUINI, W, LAMORTE, G, BIANCHI, G, POLENTARUTTI, N, ALLAVENA, P & MANTOVANI, A (1995) Receptors, signal transduction, and spectrum of action of monocyte chemotactic protein-1 and related chemokines. *J. Leukocyte Biol.* **57**, 788-794.

SPRINGER, TA (1994) Traffic siganals for lymphocyte recirculation and leukocyte emigration: The mulistap paradigm. *Cell* **76**, 301-314.

STADNICKI, A, DELACADENA, RA, SARTOR, RB, BENDER, D, KETTNER, CA, RATH, HC, ADAM, A & COLMAN, RW (1996) Selective plasma kallikrein inhibitor attenuates acute intestinal inflammation in lewis rat. *Dig. Dis. Sci.* **41**, 912-920.

STADNYK, AW (1994) Cytokine production by epithelial cells. *FASEB J.* **8**, 1041-1047.

STALNIKOWICZ, R, GOBER, K, KARMELI, F, FIOCCHI, C & RACHMILEWITZ, D (1985) (2'-5') Oligoadenylate synthatase activity in leukocytes of patients with Crohn's disease. *Gut* **26**, 556-561.

STANDIFORD, TJ, KUNKEL, SL, BASHA, MA, CHENSUE, SW, LYNCH III, JP, TOWES, GB, WESTWICK, J & STRIETER, RM (1990) Interleukin-8 gene expression by a pulmonary epithelial cell line. A model for cytokine networks in the lung. *J. Clin. Invest.* **86**, 1945-1953.

STEVENS, C, WALZ, G, SINGARAM, C, LIPMAN, ML, ZANKER, B, MUGGIA, A, ANTONIOLI, D, PEPPERCORN, MA & STROM, TB (1992) Tumor necrosis factor-a, interleukin-1b, and interleukin-6 expression in inflammatory bowel disease. *Dig. Dis. Sci.* **37**, 818-826.

STEVENS, TRJ, WINROW, VR, BLAKE, DR & RAMPTON, DS (1992) Circulating antibodies to heat-shock protein 60 in Crohn's disease and ulcerative colitis. *Clin. Exp. Immunol.* **90**, 271-274.

STRIETER, RM, KUNKEL, SL, SHOWELL, HJ, REMICK, DG, PHAN, SH, WARD, PA & MARKS, RM (1989) Endothelial cell gene expression of a neutrophil chemotactic factor by TNFa, LPS and IL-1b. *Science* **243**, 1467-1469.

STRIETER, RM, KUNKEL, SL, ARENBERG, DA, BURDICK, MD & POLVERINI, PJ (1995a) Interferon-gamma-inducible protein-10 (IP-10), A member of the C-X-C chemokine family, is an inhibitor of angiogenesis. *Biochem. Biophys. Res. Commun.* **210**, 51-57.

STRIETER, RM, POLVERINI, PJ, KUNKEL, SL, ARENBERG, DA, BURDICK, MD, KASPER, J, DZUIBA, J, VAN DAMME, J, WALZ, A, MARRIOTT, D, CHAN, S, ROCZNIAK, S & SHAANAFELT, AB (1995b) The functional role of the ELR motif in CXC chemokine-mediated angiogenesis. *J. Biol. Chem.* **270**, 27348-27357.

STUEHR, DJ & GRIFFITH, OW (1992) Mammalian nitric oxide synthases. *Adv. Enzym.* **65**, 287-346.

SUENAGA, K, YOKOYAMA, Y, OKAZAKI, K & YAMAMOTO, Y (1995) Mycobacteria in the intestine of Japanese patients with inflammatory bowel disease. *Am. J. Gastroenterol.* **90**, 76-80.

SZABO, C (1996) DNA strand breakage and activation of poly-ADP ribosyltransferase. A cytotoxic pathway triggered by peroxynitrite. *Free Radic. Biol. Med.* **21**, 855-869.

TALAL, AH & DROSSMAN, DA (1995) Psychosocial factors in inflammatory bowel disease. *Gastroenterol.Clin.North Am.* **24**, 699-716.

TAUB, DD, PROOST, P, MURPHY, WJ, ANVER, M, LONGO, DL, VAN DAMME, J & OPPENHEIM, JJ (1995a) Monocyte chemotactic protein-1 (MCP-1), -2, and -3 are chemotactic for human T lymphocytes. *J. Clin. Invest.* **95**, 1370-1376.

TAUB, DD, SAYERS, TJ, CARTER, CRD & ORTALDO, JR (1995b) a and b chemokines induce NK cell migration and enhance NK-mediated cytolysis. *J. Immunol.* **155**, 3877-3888.

TEPPERMAN, BL, BROWN, JF & WHITTLE, BJR (1993) Nitric oxide synthase induction and intestinal epithelial cell viability in rats. *Am. J. Physiol.* **265**, G214-G218.

TEPPERMAN, BL, BROWN, JF, KOROLKIEWICZ, R & WHITTLE, BJR (1994) Nitric oxide synthase activity, viability and cyclic GMP levels in rat colonic epithelial cells: Effect of endotoxin challenge. *J. Pharmacol. Exp. Ther.* **271**, 1477-1482.

THOMPSON, NP, MONTGOMERY, SM, POUNDER, RE & WAKEFIELD, AJ (1995a) Is measles vaccination a risk factor for inflammatory bowel disease. *Lancet* **345**, 1071-1074.

THOMPSON, NP, POUNDER, RE & WAKEFIELD, AJ (1995b) Perinatal and childhood risk factors for inflammatory bowel disease. A case control study. *Eur. J. Gastroenterol. Hepatol.* **7**, 385-390.

TOMASI, TBJ (1970) Structure and function of mucosal antibodies. *Annu. Rev. Med.* **21**, 281

TOYODA, H, WANG, SJ, YANG, HY, REDFORD, A, MAMALONG, D, TYAN, D, MCELREE, CK & PRESSMAN, SR (1993) Distinct association of HLA class II genes with inflammatory bowel disease. *Gastroenterology* **104**, 741-748.

TRAGNONE, A, VALPIANI, D, MIGLIO, F, ELMI, G, BAZZOCCHI, G, PIPITONE, E & LANFRANCHI, GA (1995) Dietary habits as risk factors for inflammatory bowel disease. *Eur. J. Gastroenterol. Hepatol.* **7**, 47-51.

TREJDOSIEWICZ, LK (1992) Intestinal intraepithelial lymphocytes and lymphoepithelial interactions in the human gastrointestinal mucosa. *Immunol. Letts.* **32**, 13-20.

TSAI, HH, DWARAKANATH, AD, HART, CA, MILTON, JD & RHODES, JM (1995) Increased faecal mucin sulfataseactivity in ulcerative colitis; A potential target for treatment. *Gut* **36**, 570-576.

TURNER, L, WARD, SG & WESTWICK, J (1995) RANTES-activated human T lymphocytes - a role for phosphoinositide 3-kinase. *J. Immunol.* **155**, 2437-2444.

TURNER, L, WARD, SG, SANSOM, D & WESTWICK, J (1996) A role for RANTES in T lymphocyte proliferation. *Biochem. Soc. Trans.* **24**, 5

TYSK, C, LINDBERG, E, JARNEROT, G & FLODERUSMYRHED, B (1988) Ulcerative colitis and Crohn's disease in an unselected population of monozygotic and dizygotic twins. A study of heritability and the influence of smoking. *Gut* **29**, 990-996.

TYTGAT, KMAJ, VANDERWAL, JWG, EINERHAD, AWC, BULLER, HA & DEKKER, J (1996) Quantitative analysis of MUC2 synthesis in ulcerative colitis. *Biochem. Biophys. Res. Commun.* **224**, 397-405.

UEKI, T, MIZUNO, M, UESO, T, KISO, T, NASU, J, INABA, T, KIHARA, Y, MATSUOKA, Y, OKADA, H, FUJITA, T & TSUJI, T (1996) Distribution of activated complement, C3b, and its degraded fragments, iC3b/C3dg, in the colonic mucosa of ulcerative colitis (UC). *Clin. Exp. Immunol.* **104**, 286-292.

VADDI, K & NEWTON, RC (1994) Regulation of monocyte integrin expression by b-family chemokines. *J. Immunol.* **153**, 4721-4732.

VALLANCE, P, LEONE, A, CALVER, A, COLLIER, J & MONCADA, S (1992) Accumulation of an endogenous inhibitor of nitric oxide synthesis in chronic renal failure. *Lancet* **339**, 572-575.

VANDIJK, JPM, MADRETSMA, GS, KEUSKAMP, ZJ & ZIJLSTRA, FJ (1995) Nicotine inhibits cytokine synthesis by mouse colonic mucosa. *Eur. J. Pharmacol.* **278**, R11-R12.

VIEIRA, P, DE WAAL MALEFYT, R, DANG, M-N, JOHNSON, KE, KASTELEIN, R, FIORENTINO, DF, DE VRIES, JE, RONCAROLO, M-G, MOSMANN, TR & MOORE, KW (1991) Isolation and expression of human cytokine synthesis inhibitory factor (CSIF/IL-10) cDNA clones: homology to Epstein- Barr virus open reading frame BCRF1. *Proc. Natl. Acad. Sci. USA* **88**, 1172-1176.

WAKEFIELD, AJ, DHILLON, AP, ROWLES, PM, SAWYERR, AM, PITTILO, RM, LEWIS, AAM & POUNDER, RE (1989) Pathogenesis of Crohn's disease: multifocal gastrointestinal infarction. *Lancet* **2**, 1054-1062.

WAKEFIELD, AJ, PITTILO, RM, SIM, R, COSBY, SL, STEPHENSON, JR, DHILLON, AP & POUNDER, RE (1993) Evidence of persistent measles virus infection in Crohn's disease. *J. Med. Virol.* **39**, 345-353.

WAKEFIELD, AJ (1995) Crohn's disease. The pathogenesis of a granulomatus vasculitis. A hypothesis. *Can. J. Gastroenterol.* **9**, 199-202.

WAKEFIELD, AJ, EKBOM, A, DHILLON, AP, PITTILO, RM & POUNDER, RE (1995) Crohn's disease; Pathogenesis and persistent measles virus infection. *Gastroenterology* **108**, 911-916.

WAKEFIELD, AJ, SIM, R, AKBAR, AN, POUNDER, RE & DHILLON, AP (1997) In situ immune responses in Crohn's disease: A comparison with acute and persistant measles virus infection. *J. Med. Virol.* **51**, 90-100.

WARDLE, TD, HALL, L & TURNBERG, LA (1996) Platelet activating factor: release from colonic mucosa in patients with ulcerative colitis and its effect on colonic secretion. *Gut* **38**, 355-361.

WATSON, ML, LEWIS, GP & WESTWICK, J (1988) Neutrophil stimulation by recombinant cytokines and a factor produced by IL-1-treated human synovial cell cultures. *Immunology* **65**, 567-572.

WELCH, GN, UPCHURCH, GR & LOSCALZO, J (1995) Nitric oxide as a vascular modulator. *Blood Rev.* **9**, 262-269.

WEST, GA, MATSUURA, T, LEVINE, AD, KLEIN, JS & FLOCCHI, C (1996) Interleukin 4 in inflammatory bowel disease and mucosal immune reactivity. *Gastroenterology* **110**, 1683-1695.

WHITTLE, BJR (1994) Nitric oxide in gastrointestinal physiology and pathology. In *Physiology of the gastrointestinal tract.* 3rd Ed. Ed L.R. Johnson. New York: Raven press. pp. 267-294.

WHITTLE, BJR (1995) Nitric oxide in physiology and pathology. *Histochem. J.* **27**, 727-737.

WILLOUGHBY, JMT, RAHMAN, AFMS & GREGORY, MM (1989) Chronic colitis after aeromonas infection. *Gut* **30**, 686-690.

WRIGHT, KL, WARD, SG, KOLIOS, G & WESTWICK, J (1997) Activation of phosphatidylinositol 3-kinase by interleukin-13. An inhibitory signal for inducible nitric-oxide synthase expression in epithelial cell line HT-29. *J. Biol. Chem.* **272**, 12626-12633.

YAMAMOTO, H, MORISE, K, KUSUGAMI, K, FURUSAWA, A, KONAGAYA, T, NISHIO, Y, KANEKO, H, UCHIDA, K, NAGAI, H, MITSUMA, T & NAGURA, H (1996) Abnormal neuropeptide concentrations in rectal mucosa of patients with inflammatory bowel disease. *J. Gastroenterol.* **31**, 525-532.

YANG, VW (1996) Eicosanoids and inflammatory bowel disease. *Gastroenterol. Clin. North. Am.* **25**, 317-332.

YOSHIMURA, T., TAKEYA, M., AND TAKAHASHI, K. (1991) Production and characterization of mouse monoclonal antibodies against human MCP-1. *J. Immunol.* **147**, 2229-2233.

YUE, G, SUN, FF, DUNN, C, YIN, K & WONG, PYK (1996) The 21-aminosteroid tirilazad mesylate can ameliorate inflammatory bowel disease in rats. *J. Pharmacol. Expt. Therap.* **276**, 265-270.

ZIJLSTRA, FJ, SRIVASTAVA, ED, RHODES, M, VANDIJK, APM, FOGG, F, SAMSON, HJ, COPEMAN, M, RUSSELL, MAH, FEYERABEND, C, WILLIAMS, GT, PULLAN, RD, THOMAS, GAO, VANBLANKENSTEIN, M, WILSON, JHP, ALLEN, A & RHODES, J (1994) Effect of nicotine on rectal mucus and mucosal eicosanoids. *Gut* 247-251.

ZIPSER, RD (1988) Mediators of inflammation in inflammatory bowel disease. *Dig. Dis. Sci.* **33**, 4S-5S.

ZURAWSKI, G & DE VRIES, JE (1994) Interleukin 13, an interleukin 4-like cytokine that acts on monocytes and B cells, but not on T cells. *Immunol. Today* **15**, 19-26.